Regulation of Mitogen-Activated Protein Kinase Cascade in Adult Rat Heart Preparations In Vitro

Antigone Lazou, Marie A. Bogoyevitch, Angela Clerk, Stephen J. Fuller, Christopher J. Marshall, Peter H. Sugden

Abstract The regulation of mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) was studied in freshly isolated adult rat heart preparations. In contrast to the situation in ventricular myocytes cultured from neonatal rat hearts, stimulation of MAPK activity by 1 μmol/L phorbol 12-myristate 13-acetate (PMA) was not consistently detectable in crude extracts. After fast protein liquid chromatography, MAPK isoforms $p42^{MAPK}$ and $p44^{MAPK}$ and two peaks of MEK were shown to be activated >10-fold in perfused hearts or ventricular myocytes exposed to 1 μmol/L PMA for 5 minutes. The identities of MAPK or MEK were confirmed by immunoblotting and, for MAPK, by the "in-gel" myelin basic protein phosphorylation assay. In retrogradely perfused hearts, high coronary perfusion pressure (120 mm Hg for 5 minutes), norepinephrine (50 μmol/L for 5 minutes), or isoproterenol (50 μmol/L for 5 minutes) stimulated MAPK and MEK ≈2- to 5-fold. In isolated myocytes, endothelin 1 (100 nmol/L for 5 minutes) also stimulated MAPK, but stimulation by norepinephrine or isoproterenol was difficult to detect. Immunoblotting showed that the relative abundances of MAPK and MEK protein in ventricles declined to <20% of their postpartal abundances after 50 days. This may explain the difficulties encountered in assaying the activity of MAPK in crude extracts from adult hearts. We conclude that potentially hypertrophic agonists and interventions stimulate the MAPK cascade in adult rats and suggest that the MAPK cascade may be an important intracellular signaling pathway in this response. (***Circ Res.* 1994;75:932-941.)**

Key Words • phorbol esters • endothelin • hypertension • norepinephrine • isoproterenol

In vivo, the heart responds to a variety of neurohumoral and mechanical stimuli; one facet of the response is the hypertrophy of muscle cells.[1] Myocyte hypertrophy is characterized by the accumulation of contractile proteins, induction of proto-oncogenes, and transitions in the transcription of cardiac genes.[1,2] Although the hypertrophic response has been well characterized, relatively little is known concerning the signaling mechanisms that link hypertrophic stimuli to the end response.

In the heart, the hydrolysis of membrane phosphatidylinositols is stimulated by hypertrophic agents such as endothelin (ET)[3-5] or α_1-adrenergic agonists,[6,7] potentially leading to the generation of at least two intracellular signals, inositol 1,4,5-trisphosphate and diacylglycerol.[8] The former controls Ca^{2+} movements within the cell, whereas the latter is the physiological activator of protein kinase C (PKC). Phosphatidylinositol hydrolysis in the heart is also stimulated by mechanical interventions that are potentially hypertrophic (eg, pressure overload in perfused hearts[9,10] or stretching cultured neonatal myocytes[11]). The activation of PKC has been demonstrated after stimulation of cardiac myocytes with α_1-adrenergic agonists, ET, or tumor-promoting phorbol esters[12-17] or by stretch.[18] Involvement of PKC in the hypertrophic response is implied from experiments in which phorbol 12-myristate 13-acetate (PMA) or its homologues[12,14,16,19] or plasmids encoding constitutively activated PKC[15,20] have been shown to be hypertrophic in cultured cells.

The downstream signaling events are not well understood. From studies in noncardiac systems as well as in the heart, the involvement of the mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinase [ERK]) cascade can be proposed. This cascade is implicated in many cellular growth responses and is activated by pathways coupled to either phosphatidylinositol hydrolysis/PKC activation or receptor protein tyrosine kinases. Mammalian MAPKs were originally described as serine/threonine protein kinases that are activated by various growth factors or tumor promoters in cultured cells.[21-24] They are now thought to play a critical role in a protein kinase cascade of signal transduction because they are known to phosphorylate numerous other proteins/enzymes involved in cellular regulation, thereby modulating their functions/activities.[25] Of particular relevance to cardiac hypertrophy is that certain transcription factors (c-jun, c-myc, and $p62^{TCF}$) have been identified as substrates for MAPK.[25] The activation of MAPK involves phosphorylation at both a tyrosine and a threonine residue within a conserved TEY motif in the enzyme.[22,23,26] The upstream activator of MAPK is MAPK kinase (MEK [for MAPK (or ERK) kinase]), for which the only known protein substrate is MAPK.[22] MEK is a dual-specificity protein kinase that phosphorylates MAPK on both threonine and tyrosine within a conserved TEY motif.[22] MEK is itself activated by a protein kinase by phosphorylation on two serine residues.[27,28] Current candidates for this role in mammalian cells are the proto-oncogene protein kinase c-Raf-1[29-31] and two other MEK kinases, one of which was identified by screening for mammalian

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From the Department of Cardiac Medicine, National Heart and Lung Institute, and the Chester Beatty Laboratories, Institute of Cancer Research, University of London (UK).

Correspondence to Peter H. Sugden, PhD, Department of Cardiac Medicine, National Heart and Lung Institute, Dovehouse St, London SW3 6LY, UK.

homologues of a yeast protein kinase[32] and the other identified by protein purification techniques.[33]

Recent findings using the model system of myocytes cultured from neonatal rat heart ventricles have raised the possibility that the MAPK cascade may be important in the hypertrophic response of the heart. These cells express at least two MAPK isoforms, $p42^{MAPK}$ (ERK2) and $p44^{MAPK}$ (ERK1).[34] Established hypertrophic agonists such as ET-1, phenylephrine, PMA, and fibroblast growth factors activate $p42^{MAPK}$, $p44^{MAPK}$, and MEK in these cells.[34,35] In addition, MAPK is activated in response to myocyte stretching.[18,36] Although neonatal cells respond in a hypertrophic (as opposed to a maturational) manner to such interventions, these cells are distinct in a variety of ways from myocytes in the intact adult heart. It is the adult setting to which the clinical entity of "cardiac hypertrophy" more specifically appertains. The present study has assessed the activation of MAPK and MEK by potential hypertrophic agonists/interventions in perfused hearts and in cardiac myocytes freshly isolated from adult rats. We conclude that the MAPK cascade is intact in the adult heart.

Materials and Methods

Animals and Materials

Sources of rats and reagents have been described in detail elsewhere.[17,35] Stock solutions of 50 mmol/L norepinephrine or isoproterenol were freshly prepared in 1 mmol/L ascorbate. A glutathione S-transferase (GST)–murine $p42^{MAPK}$ fusion protein was expressed in *Escherichia coli* and was used without further modification.[37] Polyclonal antiserum 124 was raised in rabbits against the peptide sequence KEKLKELIFEETAR from the C-terminal region of murine $p42^{MAPK}$.[38] Although the analogous sequence in $p44^{MAPK}$ differs from this sequence by two amino acids, some batches of this antiserum also recognized $p44^{MAPK}$.[38] Polyclonal antiserum 179 against a GST fusion protein (expressed in *E coli*) containing the rabbit MEK1 sequence[39] was raised in rabbits.

Heart Preparations and Samples

Hearts from 250- to 300-g male Sprague-Dawley rats were perfused retrogradely with Krebs-Henseleit bicarbonate-buffered saline[40] containing 10 mmol/L glucose at 37°C. Perfusion pressures are given in the figure legends. Ventricular myocytes were isolated by digestion of hearts with collagenase (Worthington type I, 1 mg/mL) as described in detail previously.[41] Myocytes from a single heart were finally resuspended in Krebs-Henseleit saline containing 20 mg/mL bovine serum albumin (BSA) and 1 mmol/L Ca^{2+}. When the activation of MAPK and MEK was to be studied by fast protein liquid chromatography (FPLC), myocytes were resuspended in a volume of buffer equal to that of the settled cells (1.0 to 1.5 mL) before exposure to PMA for 5 minutes at 37°C. When MAPK was to be studied by the "in-gel" myelin basic protein (MBP) phosphorylation assay, myocytes prepared from a single heart were resuspended to a final volume of 3 to 4 mL, and 0.5-mL aliquots were incubated for 5 minutes with agonists at 37°C. The cells were then centrifuged for 1 to 2 seconds in an Eppendorf centrifuge and then washed once with Krebs-Henseleit saline.

For the study of MAPK and MEK expression by immunoblotting, heart ventricles were removed from 1- to 50-day-old rats and frozen in liquid N_2. Samples of human ventricle were from elective terminations or autopsy and were obtained through Dr P.J.R. Barton (Department of Cardiothoracic Surgery, National Heart and Lung Institute) as described earlier.[42]

Tissue Extraction

Perfused heart ventricles were "freeze-clamped" using aluminum tongs cooled in liquid N_2 and pulverized under liquid N_2. For FPLC of MAPK and MEK, heart powder or cardiac myocytes were resuspended in 5 vol ice-cold extraction buffer containing 20 mmol/L β-glycerophosphate, 20 mmol/L NaF, 2 mmol/L EDTA, 0.2 mmol/L Na_3VO_4, 10 mmol/L benzamidine, 25 μg/mL leupeptin, 50 μg/mL phenylmethylsulfonyl fluoride (PMSF), and 0.3% (vol/vol) 2-mercaptoethanol (pH 7.5). Extracts were centrifuged for 10 minutes at 10 000*g* and 4°C, and the supernatant fractions were retained. In some cases when MAPK activity was assayed by in-gel MBP phosphorylation (see below), an equal volume of boiling sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer (10% [wt/vol] SDS, 13% [vol/vol] glycerol, 300 mmol/L Tris-HCl, 0.2% [wt/vol] bromophenol blue, and 130 mmol/L dithiothreitol [pH 6.8]) was added to a sample of supernatant fraction, followed by boiling for 5 minutes.

For immunoblotting of rat whole-heart extracts, powdered hearts were resuspended at 0°C to 4°C in 12 mmol/L Tris-HCl, 2 mmol/L EGTA, 1 mmol/L EDTA, 50 mmol/L NaF, 200 μmol/L leupeptin, 10 μmol/L *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 120 μmol/L pepstatin, 5 mmol/L dithiothreitol, and 300 μmol/L PMSF (pH 7.4). Human heart samples were provided in 1% (wt/vol) SDS. Samples were prepared for SDS-PAGE as described above except that, additionally, the DNA was sheared by repetitive passage through a 25-gauge syringe needle.

Fast Protein Liquid Chromatography

Supernatant fractions (0.2 to 0.5 mL for MAPK, 0.1 to 0.5 mL for MEK) were applied to a Mono Q HR5/5 column (Pharmacia) equilibrated with 50 mmol/L Tris-HCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol, 0.03% (wt/vol) Brij 35, 0.3 mmol/L Na_3VO_4, 1 mmol/L benzamidine, and 4 μg/mL leupeptin (final pH 7.3) at 20°C (buffer A) and a flow rate of 1 mL/min. After washing with 5 mL buffer A, proteins were eluted with a linear gradient of NaCl (0 to 300 mmol/L) in buffer A, and fractions (0.5 mL) were collected.

Assay of MAPK

MAPK was assayed by its ability to phosphorylate MBP at 30°C as described previously.[34,35] The assay mixture contained, at a final pH of 7.5 in a final volume of 50 μL, 15 mmol/L β-glycerophosphate, 0.28 mg/mL MBP, 50 mmol/L NaF, 2 mmol/L EDTA, 0.3 mmol/L Na_3VO_4, 2 μmol/L cAMP-dependent protein kinase inhibitory peptide (PKI, sequence TTYADFIASGRTGRRNAIHD), and 10 mmol/L $MgCl_2$/120 μmol/L [γ^{32}P]ATP (40 to 100 Ci/mol), with which the reaction was initiated. Reactions were terminated by spotting 40 μL of the mixture onto P81 phosphocellulose papers that were immediately immersed in ice-cold 75 mmol/L H_3PO_4. Papers were washed (once for 5 minutes and three times for 20 minutes each) in 75 mmol/L H_3PO_4 and then counted in 10 mL Fluoran HV. One unit of MAPK was that amount which catalyzed the incorporation of 1 pmol phosphate into MBP per minute.

MAPK activity was also assayed by the in-gel MBP phosphorylation assay adapted from the method of Kameshita and Fujisawa,[43] as described in detail previously.[34,35] The principle behind this method is that denatured MBP kinases migrate according to their apparent molecular masses by SDS-PAGE in gels into which MBP has been covalently incorporated. After renaturation, MBP kinases are identified by the covalent incorporation of ^{32}P from [γ-^{32}P]ATP into the MBP set in the gel. Briefly, cell extracts or column fractions (50 μg total protein loaded) were resolved in 10% (wt/vol) SDS-polyacrylamide gels formed in the presence of 0.5 mg/mL MBP. After electrophoresis, the gel was washed with 20% (vol/vol) 2-propanol in 50 mmol/L Tris-HCl (pH 8.0) and then with 5 mmol/L

2-mercaptoethanol in 50 mmol/L Tris-HCl (pH 8.0). Proteins were further denatured by washing the gel in 6 mol/L guanidine HCl in 50 mmol/L Tris-HCl (pH 8.0) and then renatured by washing in 50 mmol/L Tris-HCl (pH 8.0) containing 0.04% (vol/vol) Tween 40 and 5 mmol/L 2-mercaptoethanol at 4°C. After preincubation of the gel at 20°C for 1 hour in 40 mmol/L HEPES, 2 mmol/L dithiothreitol, and 10 mmol/L $MgCl_2$ (pH 8.0), in-gel phosphorylation of MBP was performed in 40 mmol/L HEPES, 0.5 mmol/L EGTA, 10 mmol/L $MgCl_2$, 2 μmol/L PKI, and 40 μmol/L [γ-^{32}P]ATP (5 μCi/mL, 25 μCi per gel) (pH 8.0) at 20°C for 3 hours. After extensive washing in 5% (wt/vol) trichloroacetic acid/1% (wt/vol) sodium pyrophosphate, gels were dried and autoradiographed. In-gel phosphorylation of MBP was quantified by scanning autoradiograms of the gels. Because it relies on renaturation of denatured proteins, which is unlikely ever to be complete, the in-gel MBP phosphorylation assay cannot be used to compare the activities of different MBP kinases (eg, $p42^{MAPK}$ and $p44^{MAPK}$). However, changes in the activity of a given MBP kinase can be examined.

Assay of MEK

MEK activity was assayed as described previously.[35] Mono Q fractions were sometimes diluted (usually fivefold) in buffer B containing 50 mmol/L Tris-HCl, 0.1 mmol/L EGTA, 0.1 mmol/L Na_3VO_4, 1 mg/mL BSA, and 0.1% (vol/vol) 2-mercaptoethanol (final pH adjusted to 7.5 at 20°C). Aliquots (10 μL) were incubated with a GST–murine $p42^{MAPK}$ fusion protein (5 μL at 0.05 mg/mL) in buffer A containing 0.04% (wt/vol) Brij 35 and 0.5 μmol/L okadaic acid. Activation of MAPK was then initiated by the addition of 5 μL of 80 mmol/L magnesium acetate and 0.8 mmol/L unlabeled ATP. After 30 minutes at 30°C, 25 μL of 50 mmol/L Tris-HCl, 0.8 mg/mL MBP, 0.1 mmol/L EGTA, 0.1 mmol/L Na_3VO_4, 2.25 μmol/L PKI, and 2.25 μmol/L okadaic acid (final pH 7.5 at 20°C) were added. The MAPK reaction was then initiated with 5 μL of 40 mmol/L magnesium acetate and 0.4 mmol/L ATP (0.5 μCi [γ-^{32}P]ATP) and allowed to proceed for a further 30 minutes at 30°C. The reaction was terminated by spotting 40 μL of the reaction mixture onto P81 paper and washing in H_3PO_4 as described above. The activity of endogenous MAPK in the Mono Q fractions was not sufficient to interfere with the assay of MEK. One unit of MEK is defined as the amount that, in 1 minute of the preincubation in the presence of unlabeled ATP, catalyzed the activation of exogenous GST-$p42^{MAPK}$ sufficient to transfer 1 pmol of phosphate per minute from [γ-^{32}P]ATP into MBP in the subsequent MAPK assay.

Immunoblot Analysis

Fractions from FPLC on Mono Q or whole-heart extracts were subjected to SDS-PAGE in 10% (wt/vol) polyacrylamide gels and immunoblotted as described in detail previously.[17,34,35] In some cases, fractions were concentrated ≈10-fold with Amicon microconcentrators. Primary antibodies were used at 1:1000 dilution. The secondary antibody (used at a 1:5000 dilution) was horseradish peroxidase–labeled donkey anti-rabbit immunoglobulin. Bound antibody was detected by the ECL Western blotting detection method (Amersham) according to the manufacturer's instructions, with quantification by laser densitometry when required.

Assay of Protein

Protein was measured by the Bradford assay[44] using Bio-Rad reagents.

Statistical Methods and Data Handling

Statistical significance was tested by a two-tailed Student's *t* test with a value of $P<.05$ being taken as significant. Areas under chromatography profiles were measured by using the INPLOT 4 program (GraphPad Software Inc).

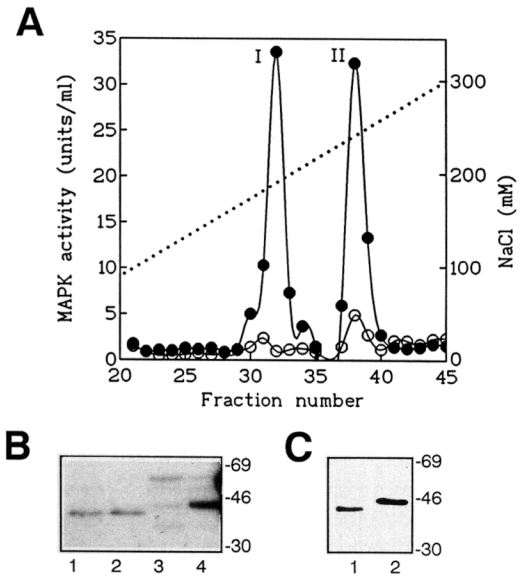

FIG 1. Fast protein liquid chromatography on Mono Q columns (Pharmacia) of mitogen-activated protein kinase (MAPK) in hearts perfused with phorbol 12-myristate 13-acetate (PMA). Control retrograde perfusions were carried out for 20 minutes at 60 mm Hg. When present, PMA was added at 15 minutes, and perfusion was continued for a further 5 minutes. A, Graph shows MAPK in supernatant fractions (0.2 mL applied to the column) from control hearts (○) and hearts perfused with 1 μmol/L PMA for 5 minutes (●) eluted from a Mono Q HR5/5 column by using linear gradients of NaCl (0 to 300 mmol/L, dotted line); 0.5-mL fractions were collected. MAPK activity was assayed as described in "Materials and Methods." Only the portion of the elution profile that revealed peaks of MAPK activity is shown. Similar elution profiles were obtained with seven separate perfused-heart preparations. B, Samples of fractions in a different Mono Q profile corresponding to two peak fractions from peak I (lanes 1 and 2) or peak II (lanes 3 and 4) were examined by the "in-gel" myelin basic protein phosphorylation assay. The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins. C, MAPK peaks I and II (lanes 1 and 2, respectively) were immunoblotted with anti-$p42^{MAPK}$ antiserum after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins.

Results

Activation of MAPK and MEK by PMA in Perfused Hearts

PMA stimulates MAPK activity in cultured neonatal rat ventricular myocytes, and this can be assayed directly in supernatant fractions of these cells using MBP as the model substrate.[34,35] In contrast, when supernatant fractions from hearts perfused with PMA (1 μmol/L for 5 minutes) were used, activation of MBP phosphorylation was low (≈1.5-fold at best) and inconsistent. However, FPLC of supernatant fractions (0.2 mL) from PMA-perfused hearts on Mono Q (Fig 1A) revealed two major peaks of MBP kinase activity eluting at ≈160 (peak I) and 220 (peak II) mmol/L NaCl. The mean stimulation of each peak (calculated from areas underneath the curves after subtraction of baseline values) was >10-fold (n=7). Recovery of MBP kinase activity following FPLC on Mono Q was typically >600% compared with activity in crude extracts.

Three criteria were used to identify the peaks of MBP kinase activity (Fig 1A) as MAPKs. First, the Mono Q

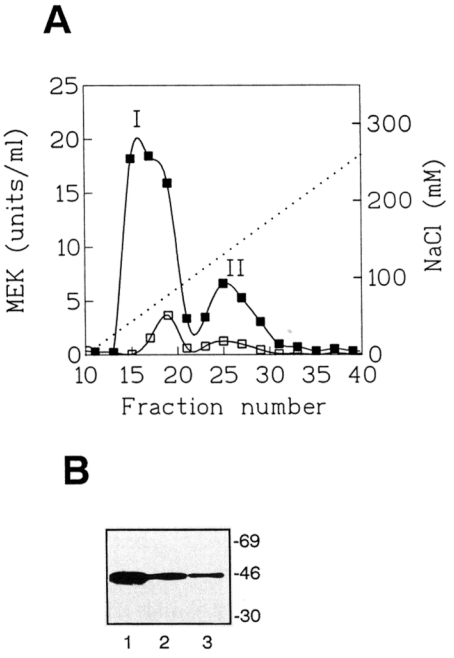

FIG 2. Fast protein liquid chromatography on Mono Q columns (Pharmacia) of mitogen-activated protein kinase kinase (MEK) in hearts perfused with phorbol 12-myristate 13-acetate (PMA). Retrograde perfusions were carried out as described in the legend to Fig 1. A, Graph shows results of MEK activities in supernatant fractions (0.1 mL applied to the column) from control hearts (□) and hearts perfused with 1 μmol/L PMA for 5 minutes (■), eluted as described from a Mono Q HR5/5 column by using linear gradients of NaCl (0 to 300 mmol/L, dotted line); 0.5-mL fractions were collected. Fractions were assayed for MEK as described in "Materials and Methods." Only the portion of the elution profile demonstrating peaks of activity is shown. Similar elution profiles were obtained with seven separate perfused-heart preparations. B, Fractions containing MEK (lane 1, peak I; lane 2, leading region of peak II; and lane 3, trailing region of peak II) were pooled, concentrated, and immunoblotted with anti–glutathione S-transferase–MEK1 antiserum after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins.

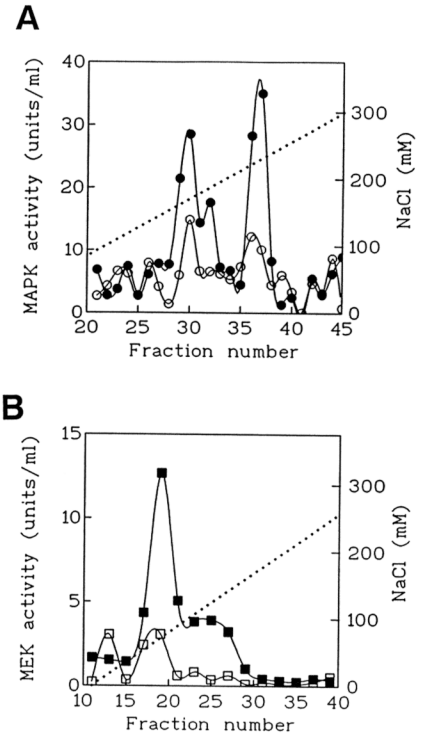

FIG 3. Graphs showing results of fast protein liquid chromatography on Mono Q columns (Pharmacia) of mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) in hearts perfused at high coronary perfusion pressures. After a 15-minute equilibration period at 60 mm Hg, hearts were perfused either at 60 mm Hg (control) or at 120 mm Hg (high coronary perfusion pressure) for a further 5 minutes. MAPK (A) and MEK (B) in supernatant fractions (0.3 mL applied to the column) from control hearts (○, A; □, B) and hearts perfused at 120 mm Hg (●, A; ■, B) were eluted and assayed as described in "Materials and Methods" and in the legends to Figs 1 and 2. The dotted line indicates the NaCl concentration. Similar elution profiles were obtained with eight (MAPK) or four (MEK) separate perfused-heart preparations.

elution profile is similar to that described for MAPK in neonatal ventricular myocytes exposed to PMA.[34,35] Second, when MBP kinase fractions were examined by using the in-gel MBP phosphorylation assay, peak I and peak II MBP kinase activities migrated at 42 and 44 kD, respectively (Fig 1B). Third, immunoblot analysis of Mono Q fractions (Fig 1C) showed immunoreactive bands at 42 kD (peak I) and 44 kD (peak II). Thus, by analogy with earlier results,[34,35] peak I is a $p42^{MAPK}$ and peak II is a $p44^{MAPK}$.

MEK in Mono Q fractions of supernatant fractions (0.1 mL loaded) of hearts perfused with 1 μmol/L PMA for 5 minutes was assayed by activation of GST-$p42^{MAPK}$ (Fig 2A). Two peaks of MEK activity eluted at 70 and 130 mmol/L NaCl. The combined stimulation of MEK activities (calculated from the total areas under the curves) was >10-fold (n=7). Fractions from Mono Q showing MEK activity were pooled, concentrated, and immunoblotted with antiserum against GST-MEK1. A single immunoreactive band of 46 kD was detected (Fig 2B).

Effects of High Coronary Perfusion Pressures on MAPK and MEK Activities in Perfused Hearts

Because systemic hypertension may induce hypertrophy of ventricular myocytes in vivo, the effect of high (120 mm Hg) coronary perfusion pressure on MAPK and MEK activities was studied in vitro. FPLC on Mono Q of soluble fractions (0.3 mL loaded) of hearts perfused at high coronary pressures revealed two major peaks of MAPK eluting at ≈160 and 220 mmol/L NaCl (Fig 3A). By analogy with Fig 1 and by in-gel MBP phosphorylation assays and immunoblotting (results not shown), these correspond to $p42^{MAPK}$ and $p44^{MAPK}$ isoforms. MAPK activities in Mono Q fractions from perfusions carried out on each of 8 days were paired. As calculated from the areas beneath the curves, high coronary perfusion pressures stimulated $p42^{MAPK}$ from 37.3±7.8 to 74.9±15.0 arbitrary units (mean±SEM, n=8, $P<.01$ by paired t test). Although $p44^{MAPK}$ activity was increased from 32.7±2.7 to 50.4±8.2 arbitrary units (mean±SEM, n=8), this was not statistically significant by paired t test. Stimulation of MEK activity by hypertensive aortic pressure was also detected after FPLC on Mono Q (Fig 3B). Two peaks of MEK activity eluted at NaCl concentrations similar to those in Fig 2A. MEK activity, calculated from the total area beneath the curve, increased from 50.7±18.0 to 114.8±24.9 arbitrary units (mean±SEM, n=4, $P<.01$ by paired t test).

The activation of MAPK by high coronary perfusion pressure detectable by FPLC on Mono Q (Fig 3A) was

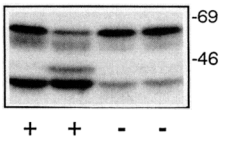

FIG 4. Effect of high coronary perfusion pressure on mitogen-activated protein kinase (MAPK) assayed by "in-gel" myelin basic protein phosphorylation. Hearts were perfused as described in the legend to Fig 3, and MAPK in the supernatant fractions was assayed by using the in-gel method (50 μg protein loaded per lane) as described in "Materials and Methods." Lanes are as follows: 1 and 2, high coronary perfusion pressure (120 mm Hg, two separate hearts); 3 and 4, control pressure (60 mm Hg, two separate hearts). The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins. The experiment was repeated with three further perfusions with similar results.

small relative to that by PMA (Fig 1A). The in-gel MBP phosphorylation assay was thus used to confirm results from FPLC. $p42^{MAPK}$ activity was stimulated in soluble fractions of hearts perfused at high coronary perfusion pressures, as shown by the increased intensity of the lowest band (Fig 4). As with FPLC on Mono Q, activation of $p44^{MAPK}$ was less consistent, as shown by inspection of the band at 44 kD running just ahead of the 46-kD marker (Fig 4). As we have observed in extracts of cultured neonatal ventricular myocytes,[35] renaturable MBP kinase activities with molecular masses in the 50- to 69-kD region (Fig 4) and in the 107-kD region (not shown) were also detected. These activities were not stimulated by high coronary perfusion pressure. The identities of these kinases are not known, but as we have observed before,[35] their activities are not stimulated by agonists that stimulate $p42^{MAPK}$ and $p44^{MAPK}$.

Activation of MAPK and MEK by PMA in Freshly Isolated Adult Ventricular Myocytes

The heart is heterogeneous in terms of its cell type, containing cells other than cardiac myocytes (nervous tissue, fibroblasts, endothelial cells, vascular smooth muscle cells, etc). Activation of MAPK and MEK by PMA was thus also studied in a more homogeneous cell population, namely, freshly isolated ventricular myocytes. Initial control experiments showed that there was no decline in the content of immunoreactive $p42^{MAPK}$ present in myocytes incubated at 37°C for 1 hour after isolation (Fig 5A). This batch of antiserum did not detect $p44^{MAPK}$ in crude extracts. Exposure of myocytes to PMA (1 μmol/L) for 5 minutes during the hour following their isolation caused phosphorylation of $p42^{MAPK}$, as demonstrated by its reduced mobility on SDS-PAGE (Fig 5B). Although $p44^{MAPK}$ was not detected by immunoblotting in this series of experiments (Fig 5A and 5B), in-gel MBP phosphorylation assays showed that activation of both $p42^{MAPK}$ and $p44^{MAPK}$ by PMA did not decline over the hour following myocyte isolation (Fig 5C). These experiments demonstrate the intracellular stability of MAPK protein and the stability of its responsiveness to activation by PMA.

Activation of MAPK and MEK was also examined by FPLC on Mono Q (Fig 6A). PMA activated two peaks of MAPK or MEK (Fig 6A), eluting at similar NaCl concentrations as in perfused hearts (Figs 1A and 2A). Both peaks of MAPK and total activity of MEK showed

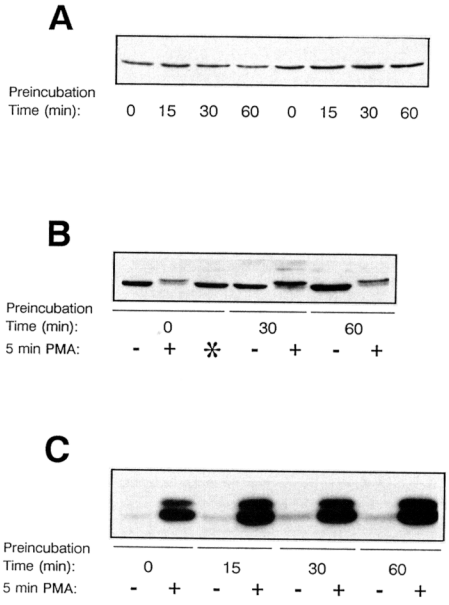

FIG 5. Stability of mitogen-activated protein kinase (MAPK) in ventricular myocytes after isolation. After isolation, myocytes were preincubated at 37°C for the times shown and subsequently prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A, MAPK isoform $p42^{MAPK}$ was immunoblotted (50 μg protein loaded per lane). B, Myocytes were exposed to phorbol 12-myristate 13-acetate (PMA, 1 μmol/L) in lanes marked "+" for 5 minutes at 37°C after preincubation, and $p42^{MAPK}$ was immunoblotted (50 μg total protein loaded per lane). PMA was omitted in lanes marked "−." In the lane marked with an asterisk, norepinephrine (50 μmol/L) replaced PMA. C, Myocytes were exposed to 1 μmol/L PMA in lanes marked "+" for 5 minutes at 37°C after preincubation, and MAPK activity was assayed by "in-gel" myelin basic protein phosphorylation (50 μg total protein loaded per lane). PMA was omitted in lanes marked "−."

>10-fold increases in activity (calculated from areas underneath the curves after subtraction of baseline values) compared with control cells (n=3). Peaks of MAPK or MEK activity were further characterized by immunoblotting. Fractions corresponding to MAPK peaks I and II were pooled, concentrated, and immunoblotted with anti-$p42^{MAPK}$ antiserum. Peak I contained bands at 42 and 44 kD, whereas peak II contained a single immunoreactive band running at slightly greater than 44 kD (Fig 6B). The probable explanation for this is that inactive $p44^{MAPK}$ coelutes with active/inactive $p42^{MAPK}$ in the peak I region. After phosphorylation and activation, $p44^{MAPK}$ eluted at a higher NaCl concentration on Mono Q and was retarded on SDS-PAGE (compare with $p42^{MAPK}$ in Fig 5B). MEK peaks I and II were also pooled, concentrated, and immunoblotted with anti–GST-MEK1 antiserum (Fig 6C). A single immunoreactive band was present at ≈45 kD.

Effects of Norepinephrine, Isoproterenol, Carbachol, ET, and Insulin on MAPK and MEK Activities in Perfused Hearts and Adult Ventricular Myocytes

In addition to PMA, a variety of other agonists stimulate the MAPK cascade in cultured neonatal rat ventricular myocytes. These include ET, phenylephrine, and fibroblast growth factors.[34,35] Norepinephrine, isoproterenol, and carbachol also stimulate MAPK and MEK in these cells (M.A. Bogoyevitch, A. Clerk, and P.H. Sugden, unpublished data). In-gel MBP phosphor-

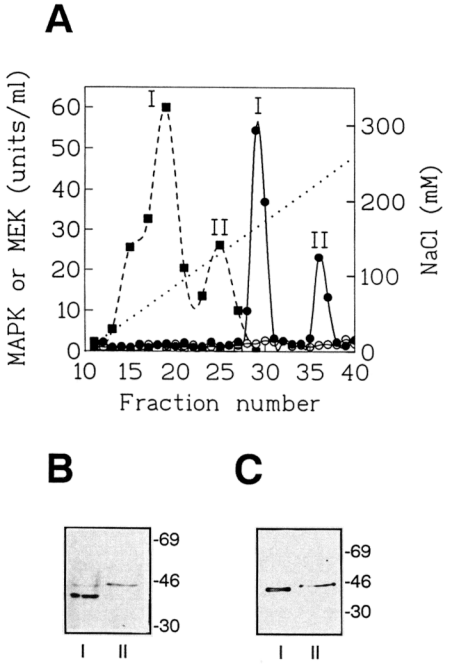

FIG 6. Fast protein liquid chromatography on Mono Q columns (Pharmacia) of mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) in freshly isolated ventricular myocytes exposed to phorbol 12-myristate 13-acetate (PMA). Cells were exposed to 1 μmol/L PMA for 5 minutes and lysed, and the supernatant fractions (0.5 mL) were applied to a Mono Q HR5/5 column. MAPK and MEK activities were eluted and assayed as described in "Materials and Methods" and in the legends to Figs 1 and 2. A, Graph shows MAPK from control cells (○, solid line) and MAPK and MEK from cells exposed to PMA (MAPK, ●, solid line; MEK, ■, broken line). Fractions assayed for MEK activity were diluted 1:5 with buffer B (see "Materials and Methods") before assay. Endogenous MAPK activity was sufficiently low so as not to interfere with the assay of MEK. Similar elution profiles were obtained with three separate preparations of myocytes. B, Fractions corresponding to MAPK peaks I and II in panel A were pooled, concentrated, and immunoblotted with anti-$p42^{MAPK}$ antiserum after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins. C, Fractions corresponding to MEK peaks I and II were pooled, concentrated, and immunoblotted with anti–glutathione S-transferase–MEK1 antiserum after SDS-PAGE. The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins.

ylation assays of the soluble fractions showed that perfusion of hearts for 5 minutes with norepinephrine (50 μmol/L) or isoproterenol (50 μmol/L) significantly stimulated both $p42^{MAPK}$ and $p44^{MAPK}$ by at least threefold, as shown by the intensities of the bands in the 42/44-kD region. Although carbachol (100 μmol/L) stimulated MAPK activities slightly (Fig 7), this result did not achieve statistical significance. As in Fig 4, other renaturable MBP kinase activities with molecular masses of >50 kD were also detectable, but their activities were not consistently increased by agonists. In addition, in two separate sets of perfusions, FPLC of soluble fractions on Mono Q (not shown) confirmed that norepinephrine (50 μmol/L) or isoproterenol (50 μmol/L) stimulated $p42^{MAPK}$, $p44^{MAPK}$, and MEK by less than fivefold, whereas carbachol (100 μmol/L) did not. Activation was calculated from areas underneath the curves after subtraction of baseline values. In a variety of cell lines, insulin stimulates MAPK.[45] However, perfusion of hearts with insulin (10 mU/mL) for 5, 10, or 120 minutes did not detectably activate MAPK or MEK. Under these conditions, protein synthesis, glucose uptake, and lactate output were stimulated appropriately,[46] demonstrating that the insulin preparation used was active (results not shown).

Because of its vasoconstrictor activity, it is not possible to perfuse hearts with ET-1. In soluble extracts of freshly isolated ventricular myocytes exposed to ET-1 (100 nmol/L), activation (twofold to threefold) of $p42^{MAPK}$ and $p44^{MAPK}$ was readily detected by in-gel MBP phosphorylation assays, as shown by the intensities of the bands in the 42/44-kD region (Fig 8). Activation was not as great as with PMA (fourfold to fivefold, Fig 8). As in Fig 4, other renaturable MBP kinase activities with molecular masses of >50 kD were also detectable, but their activities were not consistently increased by agonists. In contrast to perfused hearts (Fig 7), we were unable to demonstrate significant consistent activation of either MAPK isoform by norepinephrine (eight separate preparations of myocytes, Fig 8B) or isoproterenol (four separate preparations of myocytes, Fig 8B), although, as is apparent from Fig 8A, activation by norepinephrine was sometimes observed. Nor were we able to detect any retardation of the immunoblotted $p42^{MAPK}$ band after exposure of cells to norepinephrine (Fig 5A). In agreement with results in perfused hearts (Fig 7), carbachol did not stimulate MAPK (Fig 8).

Expression of MAPK and MEK Protein in Heart During Postnatal Development in Rat and Human

Expression of MAPK protein during maturation was investigated by immunoblotting. Relative abundance of $p42^{MAPK}$ declined rapidly after birth in rat heart (Figs 9A and 10A). Relative abundance of $p44^{MAPK}$ (the minor band above the major band in Fig 9A) also declined with age, although quantification was more difficult because of the lower signal strength. The lesser amounts of $p44^{MAPK}$ detected in comparison with $p42^{MAPK}$ may simply represent a lesser immunoreactivity of $p44^{MAPK}$ with the anti-$p42^{MAPK}$ antiserum. Quantification of the expression of $p42^{MAPK}$ protein showed that by 50 days after birth, the relative abundance of $p42^{MAPK}$ protein had declined to <15% of its value at 1 to 3 days ($P<.001$, Fig 10A). Relative abundance of MEK in rat ventricles also declined with age (Figs 9B and 10B). At 50 days after birth, relative abundance was <20% of its value at 1 to 3 days ($P<.005$). However, the fall in MEK abundance was less rapid than of $p42^{MAPK}$. At 10 days, the relative abundance of MEK was 83±6% (mean±SEM, n=3) of its value at 1 day, whereas for $p42^{MAPK}$ it was 39±13% (mean±SEM, n=3, $P<.05$ versus ratio for MEK by unpaired t test). The relative abundances of MAPK (Fig 9C) and MEK (Fig 9D) in the human heart fell after birth. The MAPK band probably represents the human homologue of rat $p42^{MAPK}$ because these proteins comigrated (results not shown). Limited availability of material precluded a more thorough analysis.

Discussion

Cardiac hypertrophy is an important physiological adaptation to an increased demand for pressure-volume work.[1,47] It is also an important clinical entity. Thus, left ventricular hypertrophy is an independent risk factor for

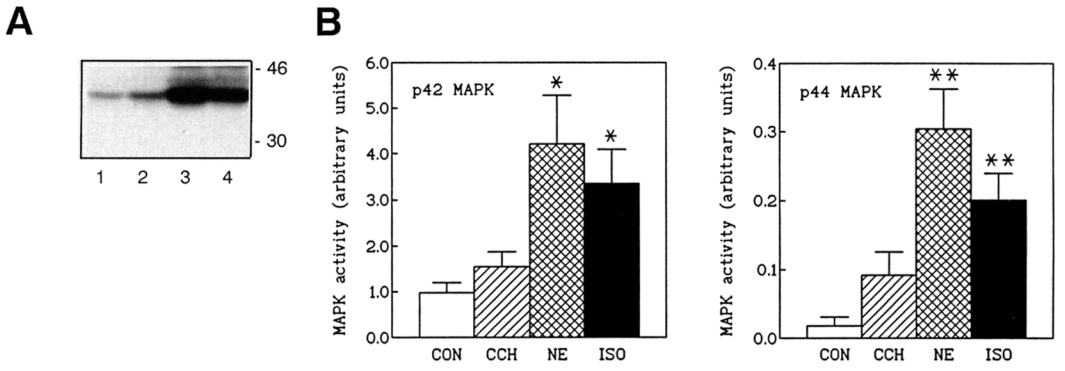

FIG 7. Activation of mitogen-activated protein kinase (MAPK) in hearts perfused with agonists assayed by "in-gel" myelin basic protein phosphorylation. A, Hearts were perfused at 60 mm Hg in the absence of agonists (control [CON], lane 1) or with 100 μmol/L carbachol (CCH, lane 2), 50 μmol/L norepinephrine (NE, lane 3), or 50 μmol/L isoproterenol (ISO, lane 4). The in-gel assay (50 μg protein loaded per lane) was carried out as described in "Materials and Methods." The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins. B, Bar graphs show results from autoradiograms quantified by laser-scanning densitometry. p42 MAPK and p44 MAPK are MAPK isoforms. Results are mean±SEM (n=3). *$P<.05$; **$P<.01$.

sudden death or other events of a cardiovascular origin.[48] Because the mammalian ventricular myocyte is a terminally differentiated cell that largely loses its ability to divide soon after birth, it adapts to increased work load principally by hypertrophy as opposed to hyperplasia. Normal maturational growth also involves an increase in the size of the cardiac myocyte. However, hypertrophy is quite distinct from maturational growth, differing primarily at the level of gene expression.[1]

The transmembrane and intracellular signaling pathways that transmit the hypertrophic stimulus to the nucleus and other organelles are not well understood and may well be multifactorial. On the basis of experiments in cultured neonatal rat ventricular myocytes, we have suggested that the MAPK cascade may be one such pathway.[34,35] However, neonatal ventricular myocytes are not typical of the adult cells, and activation of the MAPK cascade might be lost in the latter. Were this to be the case, it would clearly be difficult to implicate the MAPK cascade in the development of adult cardiac hypertrophy. Thus, it is important to establish the existence of the cascade in heart preparations from adult animals and to show that the cascade is regulated by agonists that are potentially hypertrophic.

MAPK activity can be assayed directly in crude extracts of cultured neonatal ventricular myocytes by phosphorylation of MBP. A fourfold to fivefold activation of MAPK is observed on exposure of cells to 1 μmol/L PMA for 5 minutes.[34,35] Attempts to detect stimulation of MBP phosphorylation by PMA and other potentially hypertrophic agonists in crude extracts of adult hearts were unsuccessful, so that the situation clearly differs from cultured neonatal cells. However, activation of $p42^{MAPK}$, $p44^{MAPK}$, and MEK could be clearly demonstrated in extracts of adult rat hearts after FPLC on Mono Q (Figs 1A and 2A). We confirmed that myocytic MAPK and MEK were susceptible to activation by PMA by using isolated cells (Figs 5 and 6).

In intact rats, the imposition of a hypertensive pressure overload on the heart (eg, by constriction of the ascending aorta[49]) leads to the development of cardiac hypertrophy. In humans, a leading cause of left ventricular hypertrophy is systemic hypertension.[47] It is still not clear whether this is a direct effect of pressure or is indirectly mediated by a neurohumoral pathway. By attaching neonatal ventricular myocytes to a silicone rubber sheet, a number of investigators have established that deformation of the cells by stretching induces the hypertrophic phenotype.[11,18,50,51] The mechanism may involve the release of an autocrine/paracrine factor,[18] possibly angiotensin II.[52] Stretch activates a plethora of

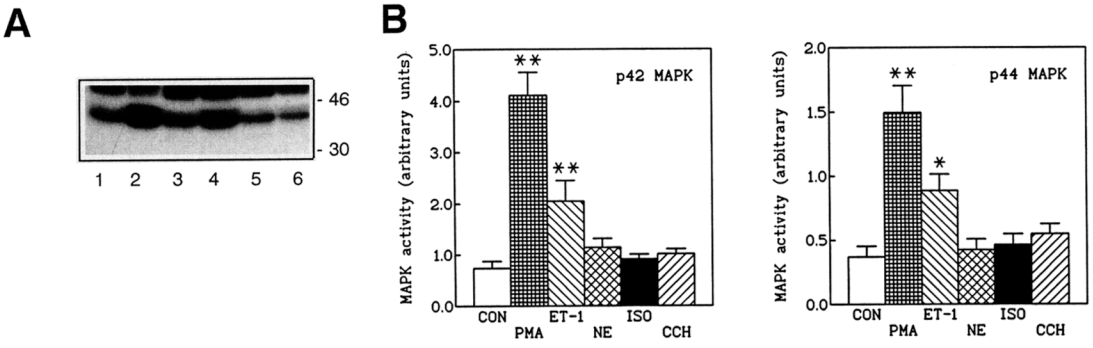

FIG 8. Activation of mitogen-activated protein kinase (MAPK) in isolated myocytes by agonists assayed by "in-gel" myelin basic protein phosphorylation. A, Cells were incubated in the absence of agonists (control [CON], lane 1) or were incubated with 1 μmol/L phorbol 12-myristate 13-acetate (PMA, lane 2), 100 nmol/L endothelin 1 (ET-1, lane 3), 50 μmol/L norepinephrine (NE, lane 4), 50 μmol/L isoproterenol (ISO, lane 5), or 100 μmol/L carbachol (CCH, lane 6). The in-gel assay (50 μg protein per lane) was carried out as described in "Materials and Methods." The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins. B, Bar graphs show results from autoradiograms quantified by laser-scanning densitometry. p42 MAPK and p44 MAPK are MAPK isoforms. Results are mean±SEM (n=4 to 8). *$P<.05$; **$P<.01$.

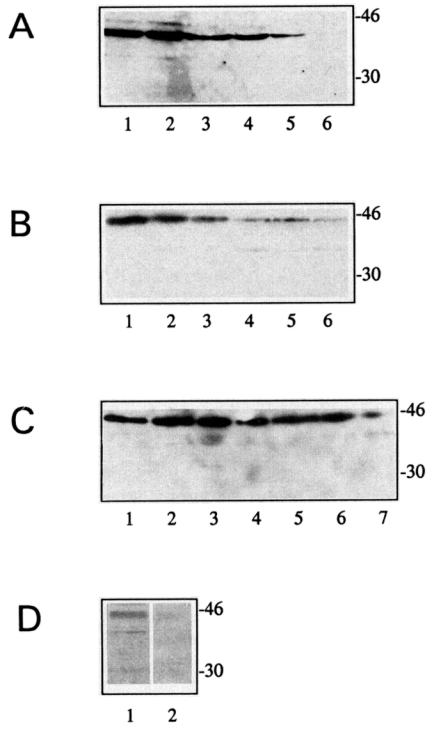

FIG 9. Immunoblotting of mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK) in heart ventricles during maturation. Immunoblotting was carried out as described in "Materials and Methods." A and B, MAPK (A) or MEK (B) immunoreactivity in rat ventricles was assayed at 1 day (lane 1), 3 days (lane 2), 10 days (lane 3), 17 days (lane 4), 26 days (lane 5), and 50 days (lane 6) after birth. For the samples from <26-day-old rats, ventricles were pooled from three to six littermates. Protein loading was 50 μg per lane. C, MAPK immunoreactivity was assayed in human heart samples after 12 weeks (lane 1), 14 weeks (lane 2), 17 weeks (lane 3), 24 weeks (lane 4), and 33 weeks (lane 5) in utero and after 15 days (lane 6) and 9 months (lane 7) after birth. Protein loading was 40 μg per lane. D, MEK immunoreactivity was assayed in human heart samples after 33 weeks in utero (lane 1) and 9 months after birth (lane 2). Protein loading was 40 μg per lane. The numbers to the right refer to the molecular masses (in kilodaltons) of the marker proteins.

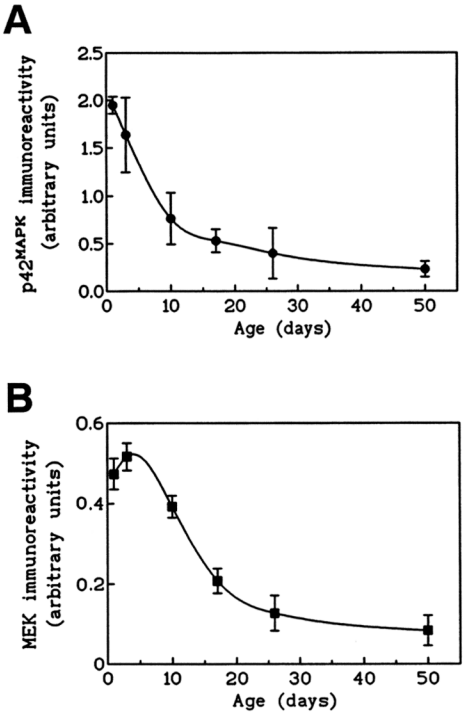

FIG 10. Graphs show relative abundance of mitogen-activated protein kinase (MAPK) isoform p42^{MAPK} and MAPK kinase (MEK) in rat heart ventricles during maturation. Immunoblots of p42^{MAPK} (A) and MEK (B) were quantified by laser densitometry. Each point represents the mean±SEM of three separate experiments.

enzymes associated with signaling pathways including phospholipases C and D (and possibly phospholipase A_2), p21 Ras, PKC, MAPK, ribosomal protein S6 kinases, protein tyrosine kinases, and the cytochrome P450 pathway of arachidonic acid metabolism.[18,36] Therefore, it was of interest to investigate whether the simple maneuver of acutely increasing coronary perfusion pressure to a hypertensive value increased the activities of MAPK cascade, since we know that this increases the rate of phosphatidylinositol hydrolysis in the perfused heart.[10] FPLC of extracts on Mono Q (Fig 3) or in-gel MBP phosphorylation assays (Fig 4) showed that high coronary perfusion pressure increased the activities of p42^{MAPK} and MEK, although increases were small (approximately a doubling) compared with PMA. Because MAPK is the only known protein substrate of MEK,[22] the activation of MEK is a strong indication of a bona fide activation of the MAPK cascade.

Two interventions that consistently induce the hypertrophic response in cultured neonatal myocytes (in addition to PMA) are ET-1 and α_1-adrenergic agonists.[1,53] In high-density cultures, β-adrenergic agonists also induce a hypertrophic response.[54] In perfused hearts, norepinephrine or isoproterenol stimulate MAPK (Fig 7) and also increase contractile activity. In contrast, no significant activation could be detected in isolated noncontracting myocytes (Fig 8) prepared under conditions that we have shown to maintain both α_1- and β_1-adrenergic responsiveness (stimulation of phosphatidylinositol hydrolysis and increase in cAMP concentrations, respectively). The failure of norepinephrine to stimulate MAPK strongly in isolated adult myocytes represents a major difference from cultured neonatal cells. The stimulation of MAPK by adrenergic agonists in the perfused heart could be related to their effects on contractile activity (as could the effect of high coronary perfusion pressure). Alternatively, the reduction in the number of nonmyocytic cells or myocyte density may be important. The latter is attractive because adrenergic agonists may stimulate nonmyocytic cells to produce myocyte growth factors.[55]

Although it is well established that isoproterenol stimulates cAMP-dependent protein kinase in heart by activating adenylyl cyclase, its activation of MAPK is a novel observation. We have detected activation of MAPK by isoproterenol in cultured neonatal myocytes, and this seems to be related to Ca^{2+} influx (M.A. Bogoyevitch, A. Clerk, and P.H. Sugden, unpublished data). Increases in intracellular Ca^{2+} concentrations stimulate MAPK in cultured fibroblasts and A431 carcinoma cells.[56]

It is difficult to assess the role of phosphatidylinositol hydrolysis and PKC in the activation of MAPK in adult myocytes. In freshly isolated adult cells, PMA activates PKC-ε (the principal PKC isoform detectable) as does epinephrine and ET-1.[17] Norepinephrine and ET-1 are both powerful stimulators of phosphatidylinositol hy-

drolysis[5,57]; however, at concentrations that maximally stimulate phosphatidylinositol hydrolysis, only ET-1 (and not norepinephrine) significantly stimulated MAPK in isolated myocytes (Fig 8). Carbachol is a moderately effective stimulator of phosphatidylinositol hydrolysis in adult myocytes[6] but does not significantly activate MAPK (Figs 7 and 8). These considerations suggest that stimulation of phosphatidylinositol hydrolysis is not in itself sufficient to produce activation of MAPK in adult cells.

The relative abundances of MAPK and MEK protein fell after birth in rat ventricles (Fig 9A and 9B and Fig 10A and 10B). This may partly explain the difficulty in detecting activation of MAPK in crude extracts of adult heart preparations compared with cultured neonatal ventricular myocytes. Preliminary FPLC on Mono Q or by SDS-PAGE was necessary. These maneuvers presumably removed substances (protein phosphatases, uncharacterized MAPK inhibitors) that interfere with direct measurement.

Our general conclusion is that the MAPK cascade is intact in adult heart preparations and is activated by interventions (PMA, mechanical stretch, ET-1, and adrenergic agonists) that may be involved in the induction of a hypertrophic phenotype.

Acknowledgments

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References

1. Chien KR, Knowlton KU, Zhu H, Chien S. Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. *FASEB J.* 1991;5:3037-3046.
2. Komuro I, Yazaki Y. Control of cardiac gene expression by mechanical stress. *Annu Rev Physiol.* 1993;55:55-75.
3. Vigne P, Lazdunski M, Frelin C. The inotropic effect of endothelin-1 on rat atria involves hydrolysis of phosphatidylinositol. *FEBS Lett.* 1989;249:143-146.
4. Shubeita HE, McDonough PM, Harris AN, Knowlton KU, Glembotski CC, Brown JH, Chien KR. Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes: a paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem.* 1990;265:20555-20562.
5. Sugden PH, Fuller SJ, Mynett JR, Hatchett RJ, Bogoyevitch MA, Sugden MC. Stimulation of adult rat ventricular myocyte protein synthesis and phosphoinositide hydrolysis by the endothelins. *Biochim Biophys Acta.* 1993;1175:327-332.
6. Brown JH, Buxton IL, Brunton LL. α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. *Circ Res.* 1985;57:532-537.
7. Steinberg SF, Kaplan LM, Inouye T, Zhang JI, Robinson RB. *Alpha*-1 adrenergic stimulation of 1,4,5-inositol trisphosphate formation in ventricular myocytes. *J Pharmacol Exp Ther.* 1989;250:1141-1148.
8. Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature.* 1993;361:315-325.
9. von Harsdorf R, Lang RE, Fullerton M, Woodcock EA. Myocardial stretch stimulates phosphatidylinositol turnover. *Circ Res.* 1989;65:494-501.
10. Bogoyevitch MA, Fuller SJ, Sugden PH. Cyclic AMP and protein synthesis in isolated adult rat heart preparations. *Am J Physiol.* 1993;265:1247-1257.
11. Komuro I, Katoh Y, Kaida T, Shibazaki Y, Kurabayashi M, Hoh E, Takaku F, Yazaki Y. Mechanical loading stimulates cell hypertrophy and specific gene expression in cultured rat cardiac myocytes: possible role of protein kinase C activation. *J Biol Chem.* 1991;266:1265-1268.
12. Henrich CJ, Simpson PC. Differential acute and chronic response of protein kinase C in cultured neonatal rat heart myocytes to α_1-adrenergic and phorbol ester stimulation. *J Mol Cell Cardiol.* 1988;20:1081-1085.
13. Kaku T, Lakatta E, Filburn C. α-Adrenergic regulation of phosphoinositide metabolism and protein kinase C in isolated cardiac myocytes. *Am J Physiol.* 1991;260:C635-C642.
14. Allo SN, McDermott PJ, Carl LL, Morgan HE. Phorbol ester stimulation of protein kinase C activity and ribosomal DNA transcription. *J Biol Chem.* 1991;266:22003-22009.
15. Shubeita HE, Martinson EA, van Bilsen M, Chien KR, Brown JH. Transcriptional activation of the cardiac myosin light chain 2 and atrial natriuretic factor genes by protein kinase C in neonatal rat ventricular myocytes. *Proc Natl Acad Sci U S A.* 1992;89:1305-1309.
16. Allo SN, Carl LL, Morgan HE. Acceleration of growth of cultured cardiomyocytes and translocation of protein kinase C. *Am J Physiol.* 1992;263:C319-C325.
17. Bogoyevitch MA, Parker PJ, Sugden PH. Characterization of protein kinase C isotype expression in adult rat heart: protein kinase C-ϵ is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. *Circ Res.* 1993;72:757-767.
18. Sadoshima J, Izumo S. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J.* 1993;12:1681-1692.
19. Dunnmon PM, Iwaki K, Henderson SA, Sen A, Chien KR. Phorbol esters induce immediate-early genes and activate cardiac gene transcription in neonatal rat myocardial cells. *J Mol Cell Cardiol.* 1990;22:901-910.
20. Kariya K, Karns LR, Simpson PC. Expression of a constitutively activated mutant of the β-isozyme of protein kinase C in cardiac myocytes stimulates the promoter of the β-myosin heavy chain isogene. *J Biol Chem.* 1991;266:10023-10026.
21. Leevers SJ, Marshall CJ. MAP kinase regulation—the oncogene connection. *Trends Cell Biol.* 1992;2:283-286.
22. Nishida E, Gotoh Y. The MAP kinase cascade is essential for diverse signalling pathways. *Trends Biochem Sci.* 1993;18:128-131.
23. Robbins DJ, Zhen E, Cheng M, Xu S, Ebert D, Cobb MH. MAP kinases ERK1 and ERK2: pleiotropic enzymes in a ubiquitous signaling network. *Adv Cancer Res.* 1994;63:93-116.
24. Blumer KJ, Johnson GL. Diversity in function and regulation of MAP kinase pathways. *Trends Biochem Sci.* 1994;19:236-240.
25. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem.* 1993;268:14553-14556.
26. Pelech SL, Sanghera JS. Mitogen-activated protein kinases: versatile transducers for cell signalling. *Trends Biochem Sci.* 1992;17:233-238.
27. Zheng C-F, Guan K-L. Activation of MEK family kinase requires phosphorylation of two conserved Ser/Thr residues. *EMBO J.* 1994;13:1123-1131.
28. Alessi DR, Saito Y, Campbell DG, Cohen P, Sithanandam G, Rapp U, Ashworth A, Marshall CJ, Cowley S. Identification of the sites in MAP kinase kinase-1 phosphorylated by $p74^{raf-1}$. *EMBO J.* 1994;13:1610-1619.
29. Kyriakis JM, App H, Zhang XP, Banerjee P, Brautigan DL, Rapp UR, Avruch J. Raf-1 activates MAP kinase kinase. *Nature.* 1992;358:417-421.
30. Hughes DA, Ashworth A, Marshall CJ. Complementation of byr1 in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase. *Nature.* 1993;364:349-352.
31. Crews CM, Erikson RL. Extracellular signals and reversible protein phosphorylation: what to Mek of it all. *Cell.* 1993;74:215-217.
32. Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science.* 1993;260:315-319.
33. Gomez N, Traverse S, Cohen P. Identification of a MAP kinase kinase kinase in phaeochromocytoma (PC12) cells. *FEBS Lett.* 1992;314:461-465.
34. Bogoyevitch MA, Glennon PE, Sugden PH. Endothelin-1, phorbol esters and phenylephrine stimulate MAP kinase activities in ventricular cardiomyocytes. *FEBS Lett.* 1993;317:271-275.
35. Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marshall CJ, Parker PJ, Sugden PH. Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes: the potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J Biol Chem.* 1994;269:1110-1119.

36. Yamazaki T, Tobe K, Hoh E, Maemura K, Kaida T, Komuro I, Tamemoto H, Kadowaki T, Nagai R, Yazaki Y. Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. *J Biol Chem.* 1993;268: 12069-12076.
37. Stokoe D, Campbell DG, Nakielny S, Hidaka H, Leevers SJ, Marshall C, Cohen P. MAPKAP kinase-2: a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.* 1992;11: 3985-3994.
38. Leevers SJ, Marshall CJ. Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *EMBO J.* 1992;11: 569-574.
39. Ashworth A, Nakielny S, Cohen P, Marshall C. The amino acid sequence of a mammalian MAP kinase kinase. *Oncogene.* 1992;7: 2555-2556.
40. Krebs HA, Henseleit K. Untersuchungen über die Harnstoff-bildung im Tierkörper. *Hoppe-Seylers Z Physiol Chem.* 1932;210: 33-66.
41. Fuller SJ, Gaitanaki CJ, Sugden PH. Effects of catecholamines on protein synthesis in cardiac myocytes and perfused hearts isolated from adult rats: stimulation of translation is mediated through the α_1-adrenoceptor. *Biochem J.* 1990;266:727-736.
42. Sasse S, Brand NJ, Kyprianou P, Dhoot GK, Wade R, Arai M, Periasamy M, Yacoub MH, Barton PJR. Troponin I gene expression during human cardiac development and in end-stage heart failure. *Circ Res.* 1993;72:932-938.
43. Kameshita I, Fujisawa H. A sensitive method for detection of calmodulin protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. *Anal Biochem.* 1989;183:139-143.
44. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-254.
45. White MF, Kahn CR. The insulin signaling system. *J Biol Chem.* 1994;269:1-4.
46. Fuller SJ, Sugden PH. Effects of insulin and adenosine deaminase on the rates of protein synthesis, glucose uptake and lactate output in the perfused working rat heart. *FEBS Lett.* 1986;201:246-250.
47. Frohlich ED, Apstein C, Chobanian AV, Devereux RB, Dustan HP, Dzau V, Fauad-Tarazi F, Horan MJ, Marcus M, Massie B, et al. The heart in hypertension. *N Engl J Med.* 1992;327:998-1008.
48. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med.* 1990;322:1561-1566.
49. Zierhut W, Zimmer H-G, Gerdes AM. Effect of angiotensin converting enzyme inhibition on pressure-induced left ventricular hypertrophy in rats. *Circ Res.* 1991;69:609-617.
50. Komuro I, Kaida T, Shibazaki Y, Kurabayashi M, Katoh Y, Hoh E, Takaku F, Yazaki Y. Stretching cardiac myocytes stimulates protooncogene expression. *J Biol Chem.* 1990;265:3595-3598.
51. Sadoshima J, Jahn L, Takahashi T, Kulik TJ, Izumo S. Molecular characterization of stretch-induced adaptation of cultured cardiac cells: an *in vitro* model of load induced cardiac hypertrophy. J Biol Chem. 1992;267:10551-10560.
52. Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell.* 1993;75:977-984.
53. Long CS, Kariya K, Karns L, Simpson PC. Sympathetic activity: modulator of myocardial hypertrophy. *J Cardiovasc Pharmacol.* 1991;17(suppl 2):S20-S24.
54. Bishopric NH, Kedes L. Adrenergic regulation of the skeletal α-actin gene promoter during myocardial cell hypertrophy. *Proc Natl Acad Sci U S A.* 1991;88:2132-2136.
55. Long CS, Henrich CJ, Simpson PC. A growth factor for cardiac myocytes is produced by cardiac nonmyocytes. *Cell Regul.* 1991;2: 1081-1095.
56. Chao T-SO, Byron KL, Lee K-M, Villereal M, Rosner RR. Activation of MAP kinases by Ca^{2+}-dependent and Ca^{2+}-independent pathways: stimulation by thapsigargin and epidermal growth factor. *J Biol Chem.* 1992;267:19876-19883.
57. Lazou A, Fuller SJ, Bogoyevitch MA, Orfali KA, Sugden PH. Characterization of the stimulation of phosphoinositide hydrolysis by α_1-adrenergic agonists in adult rat heart. *Am J Physiol.* In press.