Cellular Stresses Differentially Activate c-Jun N-terminal Protein Kinases and Extracellular Signal-regulated Protein Kinases in Cultured Ventricular Myocytes*

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Anisomycin or osmotic stress induced by sorbitol activated c-Jun N-terminal protein kinases (JNKs) in ventricular myocytes cultured from neonatal rat hearts. After 15–30 min, JNK was activated by 10–20-fold. Activation by anisomycin was transient, but that by sorbitol was sustained for at least 4 h. In-gel JNK assays confirmed activation of two retnarant JNKs of 46 and 55 kDa (J NK-46 and J NK-55, respectively). An antibody against human JNK1 immunoprecipitated J NK-46 activity. Endothelin-1, an activator of extracellular signal-regulated protein kinases (ERKs), also transiently activated J NKs by 2–5-fold after 30 min. Phorbol 12-myristate 13-acetate did not activate the J NKs although it activated ERK1 and ERK2, which phosphorylated the c-Jun transactivation domain in vitro. ATP depletion and repletion achieved by incubation in cyanide + deoxyglucose and its subsequent removal from the medium activated the ERKs but failed to activate the J NKs. Sorbitol (but not anisomycin) also stimulated the ERKs. Sorbitol-stimulated JNK activity could be resolved into three peaks by fast protein liquid chromatography on a Mono Q column. The two major peaks contained J NK-46 or J NK-55. These results demonstrate that cellular stresses differentially activate the J NKs and ERKs and that there may be “cross-talk” between these MAPK pathways.

The mammalian ventricular myocyte is a terminally differentiated cell that responds to neurohumoral or mechanical stimuli by adaptational growth in the absence of cell division (hypertrophy) (1). Increases in cell size and myofibrillar content are accompanied by transcriptional changes such as the rapid but transient expression of the immediate-early genes c-fos, c-jun, and egr-1 that encode transcription factors (1). Expression of c-fos has been used as an early marker of the hypertrophic response in the heart (2) and is controlled by multiple promoter elements that include the serum response element, the sis-inducible element, the activator protein-1 element (AP-1), and the cAMP response element (3). Regulation via the serum response element involves the phosphorylation of the ternary complex factor p62CF (or Elk-1) by the extracellular signal-regulated protein kinase (ERK) subfamily of mitogen-activated protein kinases (MAPKs) (4), whereas the sis-inducible element is involved in receptor protein tyrosine kinase signaling (3). Once expressed, Fos and other members of the Fos-related family of transcription factors heterodimerize with members of the c-J un family and transactivate at promoter regions containing AP-1 sites (5).

Rapid changes in transcriptional activity also follow the phosphorylation of c-Jun on Ser-63 and Ser-73 in its N-terminal transactivation domain (6, 7). Although ERKs will phosphorylate Ser-63 and Ser-73, another protein kinase, pp54, also phosphorylates these residues (7). This Ser/Thr protein kinase has been identified as a member of a c-J un N-terminal kinase (J NK) subfamily of MAPKs (8). J NKs are only weakly activated by growth factors or phospholipid esters, but are strongly activated by inflammatory cytokines and cellular stresses such as UV radiation, heat shock or protein synthesis inhibition (9–11).

We recently proposed an involvement of the Ras → Raf → MEK → ERK pathway in the development of hypertrophy of the ventricular myocyte (12, 13). This has been supported by studies in which transfection of ventricular myocytes with [Val12]Ha-Ras (14), active forms of c-Raf (15) or constitutively activated forms of MEK (16) produced changes in gene expression that were qualitatively similar to those observed after treatment with hypertrophic agonists (1). Transfection of myocytes with a dominant-negative form of ERK1 prevented these changes in gene expression induced by the hypertrophic agonist phenylephrine (17).

The ventricular myocyte must also adapt hypertrophically to cellular stress, such as myocardial ischemia, or reperfusion of an ischemic region (18, 19). Although myocytes in the affected region may be irreversibly damaged and subsequently die, myocytes bordering on the damaged zone increase in size to maintain the overall contractile capacity of the heart (18, 20). Given that the transcriptional activation observed during the hypertrophic response could involve activation of growth-re-

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1The abbreviations used are: AP-1, activator protein-1; DMEM, Dulbecco’s modified Eagle’s medium; DOG, 2-deoxy-D-glucose; DTT, dithiothreitol; ERK, extracellular signal-regulated (protein) kinase; ET-1, endothelin-1; FPLC, fast protein liquid chromatography; GST, glutathione S-transferase; JNK, c-Jun N-terminal protein kinase; M199, medium 199; MAPK, mitogen-activated protein kinase; MEK, MAPK (or ERK) kinase; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; SAPK, stress activated protein kinase; PKC, protein kinase C.

2We use the term mitogen-activated protein kinase (MAPK) for the family of kinases that includes the extracellular signal-regulated (protein) kinase (ERK) subfamily and the c-Jun N-terminal kinase (J NK) subfamily. ERK1 and ERK2 are also known as p44MAPK and p42MAPK, respectively. J NKs are also known as stress-activated protein kinases (SAPKs). The MAPK family also includes protein kinases that are related to the Saccharomyces cerevisiae HOG-1 osmoregulatory protein kinases.

endothelin-1; PFLC, fast protein liquid chromatography; GST, glutathione S-transferase; JNK, c-Jun N-terminal protein kinase; M199, medium 199; MAPK, mitogen-activated protein kinase; MEK, MAPK (or ERK) kinase; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; SAPK, stress activated protein kinase; PKC, protein kinase C.

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lated protein kinases additional to ERKs, we have examined the activation of isoforms of JNKs and ERKs in cultured neonatal rat ventricular myocytes exposed to cellular stresses or hypertrophic neurohumoral stimuli.

**EXPERIMENTAL PROCEDURES**

Materials—Sorbitol, anisomycin, phorbol 12-myristate 13-acetate (PMA), endothelin-1 (ET-1), phenylephrine, ionomycin, 2-deoxy-D-glucose (DOG), Medium 199 (M199), Dulbecco's modified Eagle's medium (DMEM), protease inhibitors, and bovine myelin basic protein (MBP) were from Sigma. Other tissue culture products were from Life Technologies, Ltd. Presitained molecular mass standard proteins, horseradish peroxidase-linked donkey anti-goat IgG, the enhanced chemiluminescence (ECL) Western blotting detection reagents, and autoradiography film (Hyperlfilm MP) were from Amersham International.

**Primary Culture and Treatment of Ventricular Myocytes—**Myocytes were dissociated from the ventricles of neonatal rat hearts (26). The cells were plated in DMEM/M199 (4:1 v/v) supplemented with 10% horse serum, 5% fetal calf serum, and 100 units/ml penicillin and streptomycin to remove fibroblasts. The myocytes, plated at a final density of 1.0 or 1.4 × 10^6 cells/ml on gelatin precoated 35-mm or 60-mm dishes, respectively, were confluent and spontaneously beating after 18 h. Serum was withdrawn for 24 h before the cells were further treated with either sorbitol (0.5 M), anisomycin (50 ng/ml), PMA (1 μM), ET-1 (100 μM), or phenylephrine (50 μM) in serum-free medium (DMEM/M199). For depletion of intracellular ATP, myocytes were incubated in KCN (1 mM) + DOG (20 mM) in serum-free medium (27, 28).

**Solid-phase JNK Assays—**We used a solid-phase kinase assay (10) to identify protein kinases that bind to and phosphorylate the N-terminal regulatory region of amino acids 1–135 (c-Jun(1–135)) was expressed as a glutathione S-transferase (GST) fusion protein in Escherichia coli (24, 25) (clone provided by C. J. Marshall) and was purified by glutathione-Sepharose (Pharmacia) chromatography. It was stored at -80°C in buffer containing 5% glycerol (protein concentration 50 mg/ml).

**Assay of JNK1 Immunocomplex Protein Kinase Activity**

JNKs are activated with the transient activation of JNK following osmotic shock of HeLa cells (32) or the exposure of other cell types to hypertrophic neurohumoral stimuli. JNKs may be responsible for phosphorylation of c-Jun by PKC (13). PKC isoforms of these cells (13), did not affect activation of JNKsbysorbitol (results not shown), indicating aPKC/nPKC-contrast with the transient activation of JNK following osmotic shock of HeLa cells (32) or the exposure of other cell types to hypertrophic neurohumoral stimuli.

**FPLC of c-jun Kinases and MBP Kinases—**Ventricular myocytes were lysed in Buffer A. Extracts were incubated on ice for 10 min, then centrifuged (10,000 × g, 2 × 6 min, 4°C). Supernatants were applied to a Mono Q HR5/5 column (Pharmacia) equilibrated with 50 mM Tris/HCl, 0.1 mM EGTA, 0.5 mM Na_3VO_4, and 0.1% (v/v) 2-mercaptoethanol, pH 7.0. The immunoprecipitates of JNK1 were resuspended in 30 μl of kinase Buffer containing 20 μM ATP, 30 μg of GST-c-jun(1–135), and 1–2 μCi of [γ-32P]ATP. After 20 min at 30°C with intermittent mixing, the reaction was terminated by centrifuging the immunoprecipitates and washing the pellets with SDS-PAGE sample buffer, boiled, and then separated by SDS-PAGE. Incorporation of 32P into GST-c-jun(1–135) was determined by Cerenkov counting of the c-jun band identified on Coomassie-stained gels.

**In-gel Protein Kinase Assays—**Activities of MBP kinases were assayed by the in-gel method (12, 13). When the activities c-jun(1–135) protein kinases were examined, 0.1 mg/ml GST-c-jun(1–135) replaced MBP polymerized in the gel.

**RESULTS**

Activation of JNKs in Ventricular Myocytes—**The ERK and JNK subfamilies of MAPK phosphorylate Ser-63 and Ser-73 of the N-terminal transactivation domain of c-Jun in vivo (30). ERKs do not bind c-jun with high affinity (10, 31) and JNKs may be responsible for phosphorylation of c-jun in vivo (22). We used a solid-phase kinase assay (10) to identify protein kinases that bind to and phosphorylate the N-terminal domain of c-jun.

**Exposure of ventricular myocytes to 0.5 M sorbitol or 50 ng/ml anisomycin produced 2–5-fold changes in JNK activity after 5 min, and this increased to 10–20-fold changes after 15–30 min (Fig. 1A). Activation of JNK by these agonists was also observed in cells that had not been serum-starved (results not shown). ET-1 (100 nM) activated JNK, but maximal activation (5-fold) was less than with sorbitol or anisomycin (Fig. 1A). Pretreatment with PMA (1 μM, 24 h), which depletes the classical and novel PKC isoforms of these cells (13), did not affect activation of JNKs by sorbitol (results not shown), indicating a PKC/cPKC-independent pathway of activation.

The time course of JNK activation was examined (Fig. 1A). Activation of JNK by sorbitol was sustained during a 4-h exposure (Fig. 1A). This activation following osmotic shock contrasted with the transient activation of JNK following osmotic shock of HeLa cells (32) or the exposure of other cell types to hypertrophic neurohumoral stimuli.
Regulation of JNKs and ERKs in the Heart

UV stress (8), carbachol (33), interleukin-1 (32), or tumor necrosis factor-α (34). Activation of JNK in myocytes by anisomycin or ET-1 was transient, being reversed after 180–240 min (Fig. 1A).

The protein kinases that phosphorylate c-Jun were characterized by in-gel kinase assays. No activity was detected in extracts of untreated cells (Fig. 1B) or when GST-c-Jun(un1−135) was omitted from the gels (results not shown). Following exposure to sorbitol or ET-1 for 5 min, two protein kinases with molecular masses of 46 kDa (JNK-46) and 55 kDa (JNK-55) were detected (Fig. 1B). JNK-46 was more intense than JNK-46. Activation of both JNKs followed a similar time course and, in agreement with solid-phase JNK assay described under “Experimental Procedures.” The molecular masses (kDa) of marker proteins are indicated by the numbers to the left of the panel. The arrows indicate the positions of the 55- and 46-kDa JNKs (JNK-55 and JNK-46, respectively).

Differential Activation of JNKs and ERKs by PMA—The cis-acting AP-1 element through which c-Fos/c-Jun dimers transactivate was originally recognized by its sensitivity to activation by PMA (36, 37). The mechanisms involved in PMA activation at AP-1 sites have not yet been fully elucidated. PMA activates JNKs in Jurkat cells (10) or NIH 3T3 cells (33). The reasons for this difference have not been clarified. In ventricular myocytes, PMA does not activate JNK in solid-phase kinase assays (Fig. 1A). PMA activates ERK1 and ERK2 in myocytes (12, 13, 38) and in other cells (39, 40) and these kinases also phosphorylate Ser-63 and Ser-73 of c-Jun (7). We therefore studied the extent to which PMA activated non-JNK GST-c-Jun(un1−135) kinases by comparing it with anisomycin after a 30-min exposure.

Total extracts of ventricular myocytes exposed to PMA were assayed by in-gel kinase assays using either GST-c-Jun(un1−135) (Fig. 2A) or MBP (Fig. 2B). PMA activated two protein kinases of 42 and 44–46 kDa when either MBP or GST-c-Jun(un1−135) was incubated with GST-c-Jun(un1−135) (Fig. 2B). This finding could be relevant to our failure to observe activation of JNKs by PMA (50 ng/ml) as assayed by in-gel GST-c-Jun(un1−135) kinase assays (results not shown).

In contrast to PMA, anisomycin (50 ng/ml) activated JNK-46 and JNK-55 (Fig. 2A). The JNK-55 band was less intense than that for JNK-46. Anisomycin only weakly activated ERK1 and

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Total extracts of ventricular myocytes exposed to PMA were assayed by in-gel kinase assays using either GST-c-Jun(un1−135) (Fig. 2A) or MBP (Fig. 2B). PMA activated two protein kinases of 42 and 44–46 kDa when either MBP or GST-c-Jun(un1−135) was the substrate. The ability of the 42- and 44–46-kDa protein kinases to phosphorylate MBP (Fig. 2B) suggests that ERK1 and ERK2 rather than JNKs are responsible for the GST-c-Jun(un1−135) phosphorylation. The finding that PMA did not activate JNKs as assayed using solid-phase assays (Fig. 1A) supports this conclusion. However, when total extracts of PMA-treated myocytes were incubated with GST-c-Jun(un1−135) and γ-32P]ATP, GST-c-Jun(un1−135) phosphorylation was detectable (results not shown), confirming that PMA-activated non-JNK GST-c-Jun(un1−135) kinases are present in extracts. Others have shown that ERKs copurify with phorbol ester-activated c-Jun kinases (41), supporting the conclusion that both ERKs and JNKs phosphorylate the transactivation domain of c-Jun.

It has been suggested that PMA activates JNKs in T cells only when Ca2+ influx is stimulated. Thus, PMA and the Ca2+ ionophore A23187 synergistically stimulate JNK activity (35). This finding could be relevant to our failure to observe activation of JNKs by PMA (Figs. 1A and 2A). However, simultaneous exposure of ventricular myocytes to PMA (1 μM) and the Ca2+ ionophore, ionomycin (0.1 μM) did not activate JNKs as assayed by in-gel GST-c-Jun(un1−135) kinase assays (results not shown).

In contrast to PMA, anisomycin (50 ng/ml) activated JNK-46 and JNK-55 (Fig. 2A). The JNK-55 band was less intense than that for JNK-46. Anisomycin only weakly activated ERK1 and
ERK2 as shown by in-gel phosphorylation of MBP (Fig. 2B).

Differential Activation of J NKs and ERKs by Sorbitol and Phenylephrine—The activation of ERKs and J NKs in ventricular myocytes by 0.5 mM sorbitol or 50 mM phenylephrine were compared with that by 50 ng/ml anisomycin or 1 μM PMA after 30 min of exposure (Fig. 3). Like PMA, phenylephrine is an hypertrophic agonist that activates ERK1 and ERK2 (12, 13). The activation of ERK1 and ERK2 by PMA and phenylephrine was confirmed using in-gel assays with MBP (Fig. 3A). Sorbitol (but not anisomycin) stimulated the activities of ERK1 and ERK2 (Fig. 3A). Sorbitol also reduced the mobility of both ERK isoforms (indicating their phosphorylation; Ref. 26) as assessed by SDS-PAGE and immunoblotting with anti-ERK1/ERK2 antisera (23) (results not shown). In-gel kinase assays using GST-c-j un(1–135) demonstrated the activation of J NK-46 and J NK-55 by sorbitol and anisomycin (Fig. 3B). In-gel GST-c-j un(1–135) kinase assays following precipitation of J NKs interacting with GST-c-j un(1–135) using GSH-Sepharose confirmed that both J NK-46 and J NK-55 were activated by sorbitol or anisomycin (Fig. 3C).

As described above, PMA activated two GST-c-j un(1–135) kinases that appeared to correspond with ERK1 and ERK2 (Fig. 3, compare A and B). These kinases were not recovered in the GSH-Sepharose pellet following interaction with GST-c-j un(1–135) (Fig. 3C), but remained in the supernatant (results not shown). This is consistent with the weaker interaction of ERKs with the N terminus of c-j un (31). Using PMA-activated GST-c-j un(1–135) kinases as markers, it can be concluded that the weak activation of a GST-c-j un(1–135) kinase at about 42 kDa by sorbitol (Fig. 3B) may be attributable to ERK2. Any GST-c-j un(1–135) kinase activity stimulated by sorbitol attributable to ERK1 is obscured by activation of J NK-46 (Fig. 3B). Phenylephrine (50 μM) only very weakly activated GST-c-j un(1–135) kinases (Fig. 3B) and again, based on their migration relative to PMA-stimulated GST-c-j un(1–135) kinases, these activities were probably attributable to ERK1 and ERK2. However, weak activation of J NK1 and J NK2 by phenylephrine was detected by in-gel phosphorylation of GST-c-j un(1–135) following precipitation of J NKs interacting with GSH-Sepharose (Fig. 3C).

J NK-46 Is Recognized by an Antibody against Human J NK-1—The identity of c-j un protein kinases in ventricular myocytes with previously characterized c-j un kinases (J NK-1 and J NK-2 in the rat) was investigated using an antibody directed against the C terminus of the human 46-kDa J NK1 (the human homologue of rat SAPKγ). An immunoprecipitation protocol using this agarose-conjugated antibody showed that 0.5 mM sorbitol or 50 ng/ml anisomycin activated protein kinases immunologically related to J NK1 by 6–8-fold after 15 min of exposure (results not shown) or 8–10-fold after 30 min (Fig. 4A). ET-1 (100 nM) also activated this protein kinase by 2–4-fold. Any activation by 50 μM phenylephrine was very weak. Preliminary experiments showed that immunoprecipitation was complete because a second incubation with agarose-linked antibody did not remove any further J NK activity (results not shown). Specificity of interaction was established by the finding that inclusion of the immunizing peptide prevented immunoprecipitation of J NK activity from sorbitol- or anisomycin-stimulated cells (results not shown). In-gel GST-c-j un(1–135) kinase assays showed that the immu-

![Figure 3. Activation of ERKs and J NKs by sorbitol and phenylephrine.](image)

![Figure 4. Immunoprecipitation of activated J NK by an anti-J NK1 antibody.](image)
no precipitation method measured activation of JNK-46 (Fig. 4B). JNK-55 was not recognized by the anti-JNK1 antibody and remained in the supernatant (Fig. 4B).

FPLC of GST-c-Jun(1–135) Kinases Stimulated after Exposure to Sorbitol or PMA—Activation of GST-c-Jun(1–135) kinases by exposure to 0.5 M sorbitol was greater than that observed for other agonists (Fig. 1A, and Fig. 3 and C). Sorbitol-activated GST-c-Jun(1–135) kinases were further characterized by FPLC on Mono Q columns (Fig. 5A). Three peaks of activity (J1 eluting in the isocratic wash, J2 eluting at 100–150 mM NaCl, and J3 eluting at 200–250 mM NaCl) were stimulated. No further GST-c-Jun(1–135) kinase activities were eluted at NaCl concentrations up to 1 M (results not shown). Fractions were concentrated and assayed by the in-gel kinase method using 0.1 mg/ml MBP polymerized in 10% SDS-PAGE gels as described under "Experimental Procedures." The molecular masses (kDa) of marker proteins are indicated by the numbers to the left of the panel. C, five 60-mm dishes of ventricular myocytes were exposed to control serum-free medium (C) or 1 μM PMA (M) for 5 min. Extracts were prepared and proteins separated by FPLC using a Mono Q column with elution by a linear NaCl gradient (dotted line). Fractions were assayed for JNK activity.

As shown by immunoblotting of pooled fractions (iv) with anti-ERK1/ERK2 antiserum (24), the 42- and 44-kDa MBP kinases corresponding to regions J1 or J2 (Fig. 5C). These results confirm the limited activation of the JNKs by PMA and that no novel c-Jun un kinases were activated after PMA exposure.

FPLC fractions from sorbitol-treated myocytes were also assayed for MBP kinases (Fig. 6A). Only a single asymmetric peak of MBP kinase was observed (M1, Fig. 6A). Although peaks M1 and J3 (Fig. 5A) overlapped, peak J3 consistently preceded peak M1 by 1 to 2 fractions (n = 3 separate occasions, compare Figs. 5A and 6A). MBP kinases of 55, 44, and 42 kDa were detected in pooled fractions (iv) (Fig. 5B). (The autoradiograph has been overexposed to demonstrate the limited renaturable MBP kinase present in pooled fractions (i–iii)). The 55-kDa MBP kinase may possibly correspond to a JNK-55 isofrom in pooled fractions (iv). The other JNK isofroms, including the 55-kDa isofrom in pooled fractions (iii) and the 46-kDa isofrom in pooled fractions (ii), do not utilize MBP as a substrate (Fig. 6B).

As shown by immunoblotting of pooled fractions (iv) with anti-ERK1/ERK2 antiserum (24), the 42- and 44-kDa MBP kinases correspond to ERK2 and ERK1, respectively (results not shown). We also separated extracts of PMA-treated myocytes by FPLC on MonoQ (Fig. 6C). Two peaks of PMA-stimulated MBP kinase activity were detected (Fig. 6C). These peaks co-elute with the PMA-stimulated c-Jun un kinases (Fig. 5C). Thus, PMA appears to stimulate the ERKs in the absence of significant JNK activation.

Activation of JNKs by Sorbitol Is Reversible—The mechanism of activation of JNKs by stress is unknown but may involve signaling via Ras (6, 8) and/or MEKK1 (42, 43). The
mechanisms of inactivation of the cascade have not been examined extensively. Inactivation may involve the protein phosphatase MKP-1 (44) that was originally identified as an immediate-early gene product induced after exposure of cells to heat shock or oxidative stress (reviewed in Ref. 45), but that was subsequently demonstrated to be a dual-specificity Thr/Tyr phosphatase acting upon ERK1 and ERK2 (46). Because the activation of JNKs by sorbitol was potent and sustained (Fig. 1A), we examined whether this activation was reversible, or whether irreversible cell damage that may occur under conditions of high osmotic stress induces irreversible activation of the enzymes.

We examined the time course of inactivation of JNK-46 in the ventricular myocyte following activation by 0.5 M sorbitol. Cells were treated with sorbitol for 15 min, and then followed by incubation with serum-free medium for 15, 45, or 60 min. Immunocomplex kinase assays (which detect principally JNK-46; Fig. 4B) demonstrated that JNK-46 was inactivated within 15–45 min of removal of sorbitol (Fig. 7). The inactivation of both JNK-46 and JNK-55 was confirmed using the in-gel GST-c-Jun(1–135) kinase assays (results not shown). These results suggest that sorbitol provides a potent and prolonged stimulation of the signaling pathway leading to activation of JNKs, and that JNKs can be rapidly inactivated after removal of the positive signaling components.

ATP Depletion/Repletion Activates the ERKs in the Absence of JNK—The ventricular myocyte may be exposed to cellular stresses, such as myocardial ischemia and the reperfusion of an ischemic region (18, 19). I. Schisma leads to osmotic imbalance and edema (47); reperfusion of the ischemic areas then leads to the formation of highly reactive free radicals and oxidative cell stress (48). Although the myocytes in the ischemic region may die, those bordering on this zone may increase in size to compensate for the lost contractile capacity (18, 20).

An in vitro tissue culture model of ischemia is the inhibition of ATP synthesis by exposure of myocytes to KCN + DOG (28, 49). Recently it has been shown that ERKs are activated during the recovery from metabolic inhibition (28). We exposed ventricular myocytes to concentrations of cyanide and DOG previously demonstrated to deplete intracellular ATP concentrations in these cultured cells (27). Cytosolic extracts were analyzed using the in-gel method for activation of JNKs (Fig.

![Fig. 7. Activation of JNKs by sorbitol is reversible.](image)

**Fig. 7.** Activation of JNKs by sorbitol is reversible. Individual 60-mm dishes of ventricular myocytes were exposed to serum-free medium or 0.5 M sorbitol for 15 min. The medium was then aspirated and the cells exposed to either serum-free-medium (C, dashed line) or 0.5 M sorbitol (○, solid line). Extracts were prepared then subjected to immunocomplex GST-c-Jun(1–135) kinase assays using an agarose-conjugated anti-JNK1 antibody as described under “Experimental Procedures.” This method principally assays JNK-46. JNK-46 activities (means ± S.E., n = three independent observations) are expressed relative to the activities in control cells.

**Fig. 8.** Exposure to cyanide and deoxyglucose and the subsequent removal of cyanide and deoxyglucose from the medium activates ERKs. Individual 35-mm dishes of ventricular myocytes were exposed to medium containing 1 mM KCN + 20 mM DOG for 5–120 min or to medium containing sorbitol (SRB) for 30 min. In panels B and D, the myocytes were exposed to KCN + DOG for 60 min, then exposed to fresh serum-free medium for a further 5 to 60 min. Extracts were prepared, then analyzed by the in-gel kinase method using 0.1 mg/ml GST-c-Jun(1–135) (A and B) or 0.5 mg/ml MBP (C and D) polymerized in 10% SDS-PAGE gels as described under “Experimental Procedures.” Each gel is representative of two independent experiments. The molecular masses (kDa) of marker proteins are indicated.

DISCUSSION

Signal Transduction following Exposure to Cellular Stress—The signal transduction pathways that alter gene transcription following cellular stress remain unresolved. Although UV irradiation was thought to act directly on the cell nucleus, activation of cytoplasmic protein kinase cascades has now been demonstrated (50). Two protein kinases, JNK1 and JNK2, are activated within 5 min of exposure to UV irradiation (10).

The JNKs, members of the MAPK family, are activated by phosphorylation of Thr and Tyr in a conserved Thr-Pro-Tyr sequence (reviewed in Ref. 51). JNK activity in the ventricular myocyte can be separated into three peaks by FPLC on Mono Q (Fig. 5A) that elute at a lower NaCl concentration than ERKs (Fig. 6, A and C). The relationship between the 44-kDa GST-c-Jun(1–135) kinase identified by FPLC on Mono Q (peak J in Fig. 5A) and JNK-46/JNK-55 requires further investigation. One form of the JNKs in the ventricular myocyte (JNK-46) was recognized by JNK1-specific antisera (Fig. 4, A and B). JNK-55 is presumably related to JNK-2.

Although the upstream signaling events remain unresolved, it has become clear that diverse signals, including inflammatory cytokines (9, 52), protein synthesis inhibitors (Ref. 53 and Fig. 1), ischemic reperfusion (54), and osmotic stress (Ref. 55 and Fig. 1), activate the JNK subfamily of MAPKs. The activation of the ERK subfamily of MAPKs is better understood and is mediated through receptor protein tyrosine kinases or G-protein-coupled receptors (56). In the heart, ET-1 and phenylephrine stimulate ERK activities, and this probably involves a Go-q-mediated activation of PKC (13, 26, 57). Thus PMA also
strongly activates ERKs in heart (12, 13, 38). It was of interest to study whether ET-1, phenylephrine, and PMA activated J N Ks, and equally whether activators of J N Ks (sorbitol and anisomycin) activated ERKs. Phenylephrine and PMA, although potent ERK activators (Figs. 2B and 3A), were the least effective of the J N K activators tested (Figs. 1A, 2A, 3 (B and C), 4A, and 5C). Sorbitol was the most potent activator of J N Ks (Figs. 1 (A and B) and 3 (B and C)) and also activated ERKs (Figs. 3A and 6 (A and B)). Anisomycin was a poor activator of ERKs in comparison to sorbitol or PMA (Figs. 2B and 3A). Interestingly, ET-1 was a moderately effective activator of J N Ks (Figs. 1A and 4A), as well as a powerful activator of ERKs (12, 13, 26, 57). Intraintracellular signaling by ET-1 involves both G\(_{o}\) and G\(_{q}\)-coupled pathways (58, 59). J N Ks can be activated by a G\(_{q}\)-dependent pathway activated by the m\(_{1}\)-muscarinic agonist carbachol (33) or by constitutively activated G\(_{o}\), or G\(_{13}\). The J N Ks therefore can be activated by G-protein-dependent pathways in addition to cell stress, although this may be cell- and agonist-specific.

It is important to understand the extent of cross-talk between the J N K and ERK pathways, given that an increase in the transactivating activity of cjun may be an end response of activation of both pathways. Although activation of ERKs by ET-1, phenylephrine or PMA in ventricular myocytes is maximal at 5 min (13), the maximal activation of J N Ks by any agonist (including ET-1) was slower, being maximal at 30–60 min (Figs. 1 (A and B) and 2 (A and B)). If the ERK and J N K pathways are parallel as suggested (51, 60) with cjun as a common substrate, the mechanisms leading to differential rates of activation of each pathway must be identified. This is particularly pertinent when a single agonist (e.g. ET-1 or sorbitol) is able to activate ERKs and J N Ks.

Sorbitol produced sustained activation of the J N Ks in ventricular myocytes (Fig. 1, A and B, and Fig. 7). This was not the result of an irreversible event, as the removal of sorbitol from the medium led to rapid inactivation of the J N Ks (Fig. 7). We have been unable to detect activities of the homologues of the genes encoding the J N Ks. Two attempts to detect activities of the homologues of the S. cerevisiae HOG-1 protein kinase in yeast have also been unsuccessful.

Pathophysiological Relevance of the J N K Pathway—Several studies have demonstrated that myocyte hypertrophy occurs following cardiac stress (ischemia, ischemia-reperfusion, hyperpension) (18–20, 61–63). Pathological hypertrophy involves a number of well characterized changes in gene expression (1), which are presumably mediated at the level of the nucleus through changes in the transactivating activities of transcription factors. Although neurohumoral factors cannot be discounted in the development of hypertrophy, a number of events may occur to impose cell stress and these may also be important in the hypertrophic response. Stresses include the generation of free radicals, osmotic imbalances, hypoxia, metabolic inhibition, metabolite accumulation, and changes in ion handling (64–66). Our results suggest that activation of J N Ks may be triggered by osmotic stress on ventricular myocytes, although metabolic inhibition by cyanide and DOG activated the ERKs in the absence of J N K activation (Fig. 8). We have recently shown that the J N Ks, although not activated in ischemic heart are activated upon reperfusion.4 The response of the myocyte to cellular stress is therefore complex. In an in vivo setting, it may be that parallel MAPKs are activated to produce the complete response to ischemia or the damage that follows during reperfusion of the ischemic area. The phenomenon of ischemic preconditioning, in which a short period of myocardial ischemia protects against a subsequent ischemic episode, may involve protein kinase activation (67) and parallel MAPK pathways may be important.

This study has shown that the ventricular myocyte is able to respond to cellular stress by differential activation of the J N K or ERK pathways. The ventricular myocyte in primary culture thus serves as a useful model for studying the responses to cellular stress. The exact phenotypic consequences of the J N K activation pathway must be defined by introduction of constitutively active forms of J N Ks or their upstream activators (68–70) into these cells.

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