c-Raf/MEK/ERK Pathway Controls Protein Kinase C-mediated p70S6K Activation in Adult Cardiac Muscle Cells*

Yoshihiro Iijima†, Martin Laser‡, Hirokazu Shiraishi‡, Christopher D. Willey‡, Balasubramanian Sundaravadivel‡, Lin Xu§, Paul J. McDermott¶‡, and Dhandapani Kuppuswamy¶¶

From the †Cardiology Division of the Department of Medicine, Gazes Cardiac Research Institute, Medical University of South Carolina and the ¶Ralph H. Johnson Department of Veterans Affairs Medical Center, Charleston, South Carolina 29425-2221

p70S6 kinase (S6K1) plays a pivotal role in hypertrophic cardiac growth via ribosomal biogenesis. In pressure-overloaded myocardium, we show S6K1 activation accompanied by activation of protein kinase C (PKC), c-Raf, and mitogen-activated protein kinases (MAPKs). To explore the importance of the c-Raf/MAPK kinase (MEK)/MAPK pathway, we stimulated adult feline cardiomyocytes with 12-O-tetradecanoylphorbol-13-acetate (TPA), insulin, or forskolin to activate PKC, phosphatidylinositol-3-OH kinase, or protein kinase A (PKA), respectively. These treatments resulted in S6K1 activation with Thr-389 phosphorylation as well as mammalian target of rapamycin (mTOR) and S6 protein phosphorylation. Thr-421/Ser-424 phosphorylation of S6K1 was observed predominantly in TPA-treated cells. Dominant negative c-Raf expression or a MEK1/2 inhibitor (U0126) treatment showed a profound blocking effect only on the TPA-stimulated phosphorylation of S6K1 and mTOR. Whereas p38 MAPK inhibitors exhibited only partial effect, MAPK-phosphatase-3 expression significantly blocked the TPA-stimulated phosphorylation of S6K1 and mTOR phosphorylation. Inhibition of mTOR with rapamycin blocked the Thr-389 but not the Thr-421/Ser-424 phosphorylation of S6K1. Therefore, during PKC activation, the c-Raf/MEK/extracellular signal-regulated kinase-1/2 (ERK1/2) pathway mediates both the Thr-421/Ser-424 phosphorylation. Thr-421/Ser-424 phosphorylation of S6K1 was observed predominantly in TPA-treated cells. Dominant negative c-Raf expression or a MEK1/2 inhibitor (U0126) treatment showed a profound blocking effect only on the TPA-stimulated phosphorylation of S6K1 and mTOR. Whereas p38 MAPK inhibitors exhibited only partial effect, MAPK-phosphatase-3 expression significantly blocked the TPA-stimulated phosphorylation of S6K1 and mTOR phosphorylation. Inhibition of mTOR with rapamycin blocked the Thr-389 but not the Thr-421/Ser-424 phosphorylation of S6K1. Therefore, during PKC activation, the c-Raf/MEK/extracellular signal-regulated kinase-1/2 (ERK1/2) pathway mediates both the Thr-421/Ser-424 and the Thr-389 phosphorylation in an mTOR-independent and -dependent manner, respectively. Together, our in vivo and in vitro studies indicate that the PKC/c-Raf/MEK/ERK pathway plays a major role in the S6K1 activation in hypertrophic cardiac growth.

Hypertrophic cardiac growth is a major compensatory response of the heart to an increased mechanical (hemodynamic) load in the form of either pressure or volume overload. Although this response is initially compensatory, a transition from this state to failure occurs when further growth of the heart is not sufficient to normalize the wall stress and maintain contractile function (1). Therefore, a major research interest in cardiovascular disease is to understand how the increase in hemodynamic load is transmitted intracellularly for mediating hypertrophic growth. Although the mechanical load appears to directly regulate the hypertrophic growth initiation, the signaling mechanism that connects load to such growth is not well understood.

A major cellular event during cardiac hypertrophy is increased protein synthesis (1–5). Enhanced protein synthesis may occur via accelerated protein translation, increased biogenesis of translational components, or both. A significant amount of mRNA of vertebrate cells possesses a unique 5′-terminal oligopyrimidine (5′-TOP) sequence in the 5′-untranslated region (5′-UTR), and these mRNA species generally code for specific ribosomal proteins (6, 7). Their translation is largely defrayed in part by the payment of page charges. This article must be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ To whom correspondence should be addressed: Gazes Cardiac Research Institute, 114 Doughty St., Charleston, SC 29425-2221. Tel.: 843-876-5067; Fax: 843-876-5068; E-mail: kuppusd@musc.edu.

† The abbreviations used are: 5′-TOP, 5′-terminal oligopyrimidine; UTR, untranslated region; S6K1, p70S6 kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase-1/2; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; mTOR, mammalian target of rapamycin; LV, left ventricle; RV, right ventricle; RVO, right ventricular pressure-overload; MKP-3, MAPK phosphatase-3; m.o.i., multiplicity of infection; PI3K, phosphatidylinositol-3-OH kinase; E64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; TPA, 12-O-tetradecanoylphorbol-13-acetate; BIM, bisindolylmaleimide-I; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase.
S6K1 but is predominantly present in the nucleus due to a C terminus-bound nuclear localization signal.

In the case of S6K1, complex multisite phosphorylations have been shown to occur in a sequential manner for kinase activation (22–25). At least eight different phosphorylation sites have been identified and can be divided largely into two sets (although the same numbering is used for the position of amino acids in p70 and p85 S6K1 isoforms, 23 residues should be added to convert to the numbering of the p85 isoform). One set of phosphorylation sites, which is important for the kinase activity and sensitivity to rapamycin, has sites in the linker region (Thr-389 and Ser-404 sites) and the catalytic domain (Thr-229 and Ser-371 sites) (22, 26). Thr-389 is located immediately C-terminal to the catalytic domain, and the phosphorylation at this site, which occurs during mitogenic stimulation and is rapamycin-sensitive, is important both for Thr-229 phosphorylation and increased kinase activity. The Thr-229 site is present in the activation loop of the catalytic domain, and phosphorylation at this site is mediated by the 3-phosphoinositide-dependent protein kinase 1 (27). A second set of phosphorylation sites involves four different residues in the pseudosubstrate domain: Ser-411, Ser-418, Thr-421, and Ser-424. Phosphorylation sites involve four different residues in the pseudosubstrate domain (also known as autoinhibitory domain) containing the consensus phosphorylation as well as kinase function (28). Excluding Ser-411, the main phosphorylation sites have been identified and can be divided largely into two sets: Ser-411, Ser-418, Thr-421, and Ser-424. Phosphorylation at this site, which occurs during mitogenic stimulation and is rapamycin-sensitive, is important both for Thr-229 phosphorylation and increased kinase activity. The Thr-229 site is present in the activation loop of the catalytic domain, and phosphorylation at this site is mediated by the 3-phosphoinositide-dependent protein kinase 1 (27). A second set of phosphorylation sites involves four different residues in the pseudosubstrate domain: Ser-411, Ser-418, Thr-421, and Ser-424. Phosphorylation of these residues is important for subsequent Thr-389 phosphorylation as well as kinase function (28). Excluding Ser-411, the phosphorylation of the remaining three residues is mediated independent of the rapamycin-sensitive pathway(s) (26). These phosphorylation sites in the pseudosubstrate domain (also known as autoinhibitory domain) contain the consensus “Ser/Thr-Pro” sequence and, therefore, can be phosphorylated by several members of the proline-directed protein kinases, including the MAPK and cyclin-dependent kinases (29).

At least two major signaling pathways have been described for the phosphorylation and activation of S6K1 (30, 31): a protein kinase C (PKC)-dependent pathway (32, 33), and a PKC-independent pathway that occurs via the activation of phosphatidylinositol 3-OH-kinase (PI3K) (31, 32, 34). A protein kinase A (PKA)-dependent pathway has been recently shown to activate S6K1 (35) and protein kinase B (PKB) (36), a kinase involved in the P13K-mediated S6K1 activation. Several earlier studies (32, 34, 37) demonstrate that the MAPK family members, such as ERKs, were neither necessary nor sufficient for S6K1 activation. Furthermore, an isoform of PKC (e.g. PKCδ) has been shown to associate directly with mTOR (38), indicating the possibility for S6K1 activation without the involvement of c-Raf/MEK/ERK pathway. However, recent reports (39–41) demonstrate the importance of ERK signaling for S6K1 activation under specific conditions.

In 1- to 4-h pressure-overloaded feline myocardium, our recent study (42) showed that the PKC, but not the P13K-dependent pathway, contributes significantly to S6K1 activation. In the present study, we observed that this activation was also accompanied by the activation of the c-Raf/MAPK pathway. Therefore, we used cultured adult feline cardiomyocytes or cardiocytes to explore: (i) whether c-Raf, MEK, and MAPKs are key intermediary players for PKC-mediated S6K1 activation, and (ii) whether the PKC- but not the P13K-mediated S6K1 activation requires the c-Raf/MEK/MAPK pathway. Using dominant negative c-Raf (C4B) adenovirus, we demonstrate for the first time that c-Raf is a critical downstream component for the PKC-mediated, but not the P13K or the PKA-mediated, phosphorylation of S6K1 and mTOR at their critical sites in adult feline cardiocytes. Furthermore, we demonstrate that the PKC-mediated S6K1 activation and mTOR phosphorylation require the involvement of both MEK1/2 and ERK1/2.

### Materials and Methods

**Chemicals**—Phenylmethylsulfonyl fluoride, 1, 4-dithiothreitol, and E-64 (trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane) were purchased from Roche Molecular Biochemicals GmbH (Mannheim, Germany). Aprotinin, leupeptin, sodium orthovanadate, okadaic acid, EGTA, Triton X-100, and β-glycerophosphate were obtained from Sigma Chemical Co. (St. Louis, MO); insulin was from Invitrogen (Carlsbad, CA); PD98059, U0126, SB202190, and SB203580 were from Calbiochem (La Jolla, CA).

**Antibodies**—The following antibodies were commercially obtained: anti-C-terminal S6K1 (C-18) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-phospho-Thr-389 S6K1, anti-phospho-Thr-229/Ser-236 p70 S6K1, anti-phospho-Thr-229/Thr-232 p85 S6K1, anti-phospho-Thr-229/Thr-232 p85 MAPK, anti-phospho-Thr-183/Tyr-185 S6K1, anti-phospho-Thr-229 p85 MAPK, and anti-phospho-Thr-389 p85 MAPK from Cell Signaling Technology Inc. (Beverly, MA); anti-c-Raf from Transduction Laboratory (Lexington, KY).

**Animal Model**—Adult cats weighing ~3 kg were used for right ventricular pressure-overload (RVPO) by partial occlusion of the pulmonary artery, as we described previously (43, 44). Briefly, cats underwent partial pulmonary artery occlusion by external banding for 24 and 48 h or insertion of a balloon catheter for 1 and 4 h. Systemic arterial pressure remained the same whereas the pulmonary arterial pressure was at least doubled. The left ventricle (LV) from each cat served as the same animal internal control for pressure-overloaded RV. Additional control (LV) and unoperated (LV) were obtained from sham-operated cats by thoracotomy and pericardiotomy without any arterial occlusions. The care of the animals and all experiments were conducted in accordance with the institutional guidelines of Medical University of South Carolina.

**Adult Cardiocyte Culture Model**—Adult feline cardiocytes were isolated from normal cats and cultured on laminin-coated four-well culture tissue culture-treated plates (45). Isolated cardiocytes were suspended in a 1.8 mM Ca²⁺ containing mitogen-free M-199 medium at pH 7.4. Cells were plated at a density of 1.5 × 10⁵ cells/well and cultured at 37 °C in humidified air with 5% CO₂.

**Recombinant Adenoviruses for Dominant Negative c-Raf Expression**—c-Raf dominant negative mutant plasmid (RSV-Raf-C4B) was kindly provided by Dr. M. Abdellatif at the Baylor College Medicine. The plasmid was derived by fusing the N-terminal regulatory domain of c-Raf to the C-terminal antigenic region of B-Raf (46). The dominant negative effect is attributed to a cysteine finger domain in the N-terminal regulatory domain (C4), which interacts with upstream factors resulting in the loss of endogenous c-Raf activation.

RSV-Raf-C4B was cloned into adenovirus shuttle plasmid pAd.CMV-Link.1 (47). Each cDNA insert was subcloned into the multiple cloning site of pAd.CMV plasmid and isolated cardiocytes were suspended in 5 × 10⁵ cells/well and cultured at 37 °C in humidified air with 5% CO₂. The purified plasmid was modified previously to produce a high level of expression by placing the constitutively active CMV promoter on the 5'-end of the multiple cloning sites and the SV40 polyadenylation signal on the 3'-end. As a test to demonstrate constitutive expression of the cDNA insert, the shuttle plasmid was transiently transfected into human 293 kidney cells and protein expression monitored by Western blotting. The purified shuttle plasmid was digested with the restriction enzymes NheI and NarI to obtain the “rescue” fragment. The fragment was then purified on agarose gel, and 2 μg of purified rescue fragment was used for homologous recombination.

The shuttle plasmid contained linker arms flanking either side of the cDNA insert that consists of adenovirus-5 sequences from 0 to 1 map unit and 9 to 16 map units, respectively. The plasmid was modified previously to produce a high level of expression by placing the constitutively active CMV promoter on the 5'-end of the multiple cloning sites and the SV40 polyadenylation signal on the 3'-end. As a test to demonstrate constitutive expression of the cDNA insert, the shuttle plasmid was transiently transfected into human 293 kidney cells and protein expression monitored by Western blotting. The purified shuttle plasmid was digested with the restriction enzymes NheI and NarI to obtain the “rescue” fragment. The fragment was then purified on agarose gel, and 2 μg of purified rescue fragment was used for homologous recombination.

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After large-scale preparation, adenoviruses were purified by CsCl gradient centrifugation, dialyzed, and titered by plaque assay (48, 49). Adenovirus for mitogen-activated protein kinase phosphatase-3 (MKP-3), generated using similar protocol, was obtained from Dr. Donald R. Menick's laboratory.

**Stimulation of S6K1 in Cultured Cardiocytes**—Freshly isolated adult feline cardiocytes were cultured overnight and stimulated with 200 nM TPA, 100 nM insulin, or 1 μM forskolin in the presence or absence of various pharmacological agents for indicated periods of time. Stock solutions for TPA and forskolin were prepared in Me2SO, and Me2SO-treated cardiocytes were used as controls for these experiments. For treatment with pharmacological inhibitors, cardiocytes were preincubated for 30 min with the inhibitors and then the cells were stimulated with TPA, insulin, or forskolin. For the adenoviral expression, freshly isolated cardiocytes were plated on laminin-coated trays and incubated for 4 h prior to infection. Cells were then incubated overnight in serum-free M-199 media containing the adenovirus at m.o.i. (multiplicity of infection) levels of 2 and 10 for MKP-3 and 250 for CAB. Cells infected with an equal m.o.i. of β-galactosidase adenovirus served as control. The media was replaced with serum-free M199 media, and cells were incubated for an additional 24 h before agonist stimulation.

**Western Blotting**—Triton X-100 soluble and insoluble samples were prepared as we previously described (50) with a few minor modifications. Briefly, following the stimulation, cardiocytes were extracted with lysis buffer (30 mM Tris-HCl, pH 7.4, 2% Triton X-100, 10 mM β-glycerolphosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μM E-64, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.02 mM okadaic acid, 0.5 mM EGTA). The cell lysate was centrifuged at 160,000 x g for 30 min, and the protein concentration in the supernatant was measured using BCA reagent (Pierce) reagent. The supernatant was then boiled with SDS-sample buffer and used as detergent-soluble fraction. The pellet obtained after centrifugation was resuspended in SDS-sample buffer, boiled for 5 min, and centrifuged for 5 min at room temperature to obtain detergent-insoluble fraction. The protein concentration in each sample was determined using BCA reagent (Pierce) and adjusted for comparison.

20 μl of detergent-lysed substractions (or insoluble fraction in the case of S6 protein detection) was resolved by SDS-PAGE, and the proteins were transferred electrophoretically to Immobilon-P membranes (Millipore Corp., Bedford, MA). The membranes were blocked for 1 h using 10% milk in TBST buffer (10 mM Tris, 0.1 M NaCl, 0.1% Tween 20, pH 7.4). Blots were incubated with the primary antibodies in TBST buffer overnight at 4 °C with gentle agitation. Following the incubation with the primary antibodies, blots were washed three times with TBST buffer each for 5 min and incubated with appropriate horseradish peroxidase-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) in TBST buffer for 1 h at room temperature. After washing the blots, the proteins were detected by enhanced chemiluminescence (Renaissance, PerkinElmer Life Sciences, MA).

**RESULTS**

**S6K1 Activation in Pressure Overloaded Myocardium**—The purpose of this study was to demonstrate that the PKC-mediated S6K1 activation in adult cardiocytes requires the activation of the c-Raf/MEK/ERK pathway. To test this possibility, we analyzed whether the S6K1 phosphorylation/activation in pressure-overloaded feline myocardium is accompanied by the activation of MAPK family members (ERK1/2, p38 MAPK, and JNKs), c-Raf, and PKC. The activation of all these kinases can be analyzed by taking three different approaches: (i) Western blot analysis using regular antibodies to observe changes in the mobility on SDS-PAGE, which is indicative of distinct phosphorylation of these kinases upon activation, (ii) Western blot analysis using phospho-specific antibodies to determine the phosphorylation of critical residues necessary for kinase activation, and (iii) immune complex kinase assays. In the present study, we undertook the first two approaches.

Right ventricular pressure overload was induced for time periods of 1–48 h by pulmonary artery occlusion. Sham-operated control cats underwent similar surgical interventions without occluding the pulmonary artery. Compared with the sham control ventricles (LV or RV) or unloaded same animal LV controls, 1-h pressure-overloaded RVs exhibited a dramatic change in the mobility of both the S6K isoforms (p70 and p85) during SDS-PAGE separation (Fig. 1A). This retarded mobility (band shifting) was also seen in 4- and 24-h pressure overloaded RV, and returned partially to the control levels in 48 h RV. We used phospho-specific antibodies that detect either the Thr-389-phosphorylated or the Thr-421/Ser-424-phosphorylated (simultaneously) S6K1 isoforms. Thr-389 and Thr-421/Ser-424 phosphorylation of the p70 S6K isoform was either almost absent or present at very low levels in unloaded control LVs and RVs. However, 1-h pressure-overloaded RVs exhibited a robust increase in phosphorylation of all three sites. Such increased phosphorylation was also observed in 4-, 24-, and...
48-h pressure-overloaded RVs. In the case of the p85 isoform, Thr-389 phosphorylation was significant in 4- and 24-h pressure-overloaded myocardium, whereas for the Thr-421/Ser-424 sites, significant phosphorylation was observed as early as 1 h and persisted up to at least 48 h of pressure overloading. The pattern of S6K1 activation matches precisely with our earlier studies performed under similar conditions (42). Overall, these data demonstrate once again that S6K1 is activated to a substantial level as early as 1 h of pressure overloading of the myocardium, and the activation is sustained for at least 24 h. Interestingly, the S6K1 activation is also accompanied by an increased mTOR phosphorylation at the Ser-2448 site. Such enhanced phosphorylation, which is indicative of mTOR activation (51), was observed as early as 1 h of pressure overload and matches the S6K1 activation time course.

Next, we analyzed whether a signaling pathway that includes the activation of PKC, c-Raf, and the MAPK family members accompanies the S6K1 activation process. The activation of MAPK family members and PKC was determined by Western blot analysis using phospho-specific antibodies whereas c-Raf activation was analyzed by its retarded electrophoretic mobility during SDS-PAGE separation combined with Western blot detection. Analysis using phospho-specific antibodies for extracellular signal-regulated kinase (ERK isoforms, 42 and 44 kDa), p38 MAPK, and c-Jun N-terminal kinase (JNK isoforms, 42 and 60 kDa) showed a clear activation of all of these family members in 1- to 4-h pressure-overloaded right ventricular samples (Fig. 1B). Whereas both of the ERK isoforms remained active up to 48 h, p38 MAPK and JNK showed a decline in phosphorylation in 24- and 48-h pressure-overloaded myocardium following their initial activation. Furthermore, pressure overloading for 1 h or more resulted in a retarded electrophoretic mobility of c-Raf (disappearance of the lower band) during SDS-PAGE separation, which is indicative of phosphorylation and activation as reported previously (52). To demonstrate PKC activation, we used a commercially available phospho-specific pan PKC antibody. Although the antibody detected protein bands even in the unloaded controls (lower most band is present both in LV or RV of control cat sample), pressure-overloaded RV samples, relative to the unloaded (same animal) LV control, exhibited one or more newly phosphorylated PKC isoforms (upper bands) indicative of their activation. The time course of activation of the PKC/c-Raf/MAPK pathway and S6K1 is very similar.

**Agonist-stimulated S6K1 Activation in Adult Cardiocytes**—Cardiocytes cultured for 24 h were stimulated with 200 nM TPA, 100 nM insulin, or 1 μM forskolin to activate PKC, PKA, or PKA, respectively. Treatment with all three agents activated both p70 and p85 isoforms (S6K1) within 30 min as evidenced by the retarded electrophoretic mobility during SDS-PAGE separation (Fig. 2A). Furthermore, studies performed with phospho-specific antibodies demonstrated that stimulation of cardiocytes with all three agents resulted in the Thr-389 phosphorylation of both the S6K1 isoforms (p70 and p85). This phosphorylation was observed as early as 8 min and peaked within 30 min of treatment. However, Thr-421/Ser-424 phosphorylation was significantly increased over control only in TPA-treated cells. Furthermore, TPA-stimulated phosphorylation at Thr-421/Ser-424 sites (8 min) was found to occur prior to the phosphorylation at the Thr-389 residue (15 min) and remained higher up to 60 min. This phosphorylation pattern of the Thr-389 and Thr-421/Ser-424 residues in S6K1 isoforms following the TPA treatment appeared similar to the changes observed in pressure-overloaded myocardium (Fig. 1A).

Next, we analyzed whether the agonist-stimulated S6K1 activation was accompanied by the activation of one or more c-Raf/MEK/MAPK pathways. Compared with the untreated or Me_SO-treated controls, TPA-treated cardiocytes exhibited a substantial phosphorylation/activation of ERK1/2 (both the p42 and p44 isoforms) and p38 MAPK (Fig. 2B). However, TPA treatment did not result in a significant activation of JNK family kinases (both p42 and p60 isoforms). In the Western blot performed for JNK, a low level protein band in the TPA panel actually corresponds to ERK1/2 and not JNK. Apparently, this might be due to cross-reactivity of the phospho-specific JNK antibody. In the case of the other two stimulants, there were no significant changes observed for all the MAPK family members following the insulin treatment, although forskolin treatment resulted in p38 MAPK activation. Furthermore, c-Raf showed retarded electrophoretic mobility only in TPA-treated cardiocytes (Fig. 2B, TPA panel), and this observation was similar to that seen in pressure-overloaded myocardium. However, all three stimulants, similar to their effect on S6K1, caused phosphorylation of mTOR at the Ser-2448 site, indicative of its activation (51). Overall, these results demonstrate that only in the TPA-stimulated cardiocytes, S6K1 activation is accompanied by the activation of c-Raf/ERK signaling pathway and that
this activation pattern matches the observation in \textit{in vivo} pressure-overloaded myocardium.

\textbf{Specificity of TPA and Insulin-stimulated S6K1 Activation}—We analyzed whether TPA, insulin, and forskolin activate S6K1 via stimulating PKC, PI3K, and PKA, respectively. For this, we used specific pharmacological inhibitors, bisindolylmaleimide-I (BIM) and wortmannin to block PKC and PI3K, respectively. Pretreatment of cardiocytes with 5 \textmu M BIM resulted in the loss of the TPA-stimulated but not the insulin- or forskolin-stimulated S6K1 phosphorylation/activation (Fig. 3). On the other hand, 100 nM wortmannin had no effect on TPA- and forskolin-stimulated S6K1 activation but significantly blocked the insulin-stimulated S6K1 activation, as evidenced by a faster electrophoretic mobility and reduced Thr-389 phosphorylation. These data indicate that S6K1 activation during TPA, insulin, and forskolin stimulation is mediated via PKC, PI3K, and PKA pathways, respectively.

\textbf{Effect of Dominant Negative c-Raf on S6K1 Activation}—A major interest of this study is to demonstrate that, during PKC-mediated S6K1 activation, c-Raf plays a critical intermediate role. Both in pressure-overloaded myocardium and in TPA-stimulated adult cardiocytes, S6K1 activation was accompanied by a retarded mobility of c-Raf during SDS-PAGE separation, indicative of its kinase activation.

We constructed a recombinant adenovirus, because the conventional transfection is not possible in adult cardiocytes, to facilitate the expression of dominant negative c-Raf mutant (C4B). Adenovirus harboring the \(\beta\)-galactosidase gene was used as a control virus. Cardiocytes were infected with 250 m.o.i. of adenovirus, a concentration determined to block the c-Raf-mediated effect. In C4B adenovirus-infected cardiocytes, but not in \(\beta\)-galactosidase adenovirus-infected cardiocytes, C4B protein was expressed in substantial amounts (Fig. 4A), at least 20-fold higher than the endogenous c-Raf level (the same antibody was used to detect the 32-kDa C4B and 65-kDa c-Raf). TPA treatment of control or \(\beta\)-galactosidase-expressing cells resulted in the retarded electrophoretic mobility of c-Raf, as observed earlier (Fig. 2B). However, such effect was completely lost in the case of C4B-expressing cells and showed faster migration, indicating the loss of c-Raf activation (Fig. 4A). c-Raf activation was low in insulin- and forskolin-treated cardiocytes, and the expression of dominant negative c-Raf blocked even the low level activity of c-Raf as indicated by the faster mobility of this kinase during SDS-PAGE separation.

Next, we analyzed whether the expression of dominant negative c-Raf (C4B) results in the loss of MAPK activation. Compared with the control and insulin- and forskolin-treated cells,
phosphorylation at Thr-389 and Thr-421/Ser-424 sites). How-
ever, unlike the C4B that blocked only the TPA effect, the
forskolin stimulation. Therefore, all these studies clearly demonstrate that the dom-
ninant negative c-Raf specifically blocks the TPA-stimulated
activation. The TPA-stimulated activation (Fig. 2B), its activation was low in the present experiment.
Furthermore, forskolin stimulation, but not TPA or insulin-
stimulation, lowered the c-Raf level in these long term cultured
(a total of 3 days) cardiocytes, used for adenoviral infection.

We also analyzed the phosphorylation status of mTOR using
a Ser-2448 phosphorylation state-specific antibody. All three
stimulants phosphorylated mTOR at the Ser-2448 site (Fig.
4A). C4B expression specifically blocks the TPA-stimulated but
not the insulin- or forskolin-stimulated mTOR phosphoryla-
tion. Therefore, c-Raf activity is required only for the TPA-
stimulated mTOR activation.

Because the expression of dominant negative c-Raf resulted
in the loss of the c-Raf/MAPK pathway, we next analyzed the importance of this pathway for S6K1 activation. Expression of
dominant negative c-Raf (C4B) almost completely blocked the
TPA-stimulated changes in the electrophoretic mobility of
S6K1 isoforms (both p70 and p85), as well as blocked the
phosphorylation at Thr-389 and Thr-421/Ser-424 sites (Fig.
4B). Importantly, this effect is very specific to TPA-stimulated S6K1 activation, because the insulin-stimulated S6K1 activa-
tion is unaffected under these conditions. Similarly, in forsko-
lin-stimulated cardiocytes, the retarded electrophoretic mobil-
ity was not affected by the expression of C4B, although a low
level reduction was observed in Thr-389 phosphorylation.

To demonstrate further that C4B expression blocks S6K1
activation in TPA-stimulated cells, we analyzed the phospho-
ylation state of 40 S ribosomal S6 protein, a specific target of
S6K1 isoforms (9–12). Stimulation of cardiocytes with all the
stimulants resulted in a substantial increase in the level of
phosphorylated S6 protein (Fig. 4B). However, C4B, but not the control (β-galactosidase) adenovirus-infected cardiocytes,
showed a complete loss of TPA-stimulated S6 protein phospho-
ylation. Similar to the effect on S6K1, S6 protein phosphoryla-
tion was unaffected during insulin or forskolin stimulation.
Therefore, all these studies clearly demonstrate that the dom-
ninant negative c-Raf specifically blocks the TPA-stimulated
(PKC-mediated) S6K1 activation and S6 protein phosphoryla-
tion and that these events are unaffected during insulin or forskolin stimulation.

Effect of MEK1/2 Inhibitor (U0126) on S6K1 Activation—A
major downstream target of c-Raf is mitogen-activated protein
kinase kinase/ERK kinase (MEK1/2), which is an immediate
upstream activator of ERK1/2. We employed U0126, a specific
inhibitor of MEK1/2, and analyzed whether pretreatment with
this drug blocks TPA-stimulated S6K1 activation. The effect of
U0126 on TPA-stimulated S6K1 activation mirrors the C4B
(dominant negative c-Raf) effect. The TPA-stimulated activa-
tion of both the S6K1 isoforms (p70 and p85), as measured in
terms of either band-shifting or phosphorylation at Thr-389
and Thr-421/Ser-424 sites, was significantly blocked when cardi-
oocytes were preincubated with 10 μM U0126 (Fig. 5). How-
ever, unlike the C4B that blocked only the TPA effect, the
U0126 treatment showed a low level blocking effect on the
insulin-stimulated S6K1 activation (as evidenced by the partial
reversal of both the electrophoretic mobility and the loss of
phosphorylation at Thr-389 and Thr-421/Ser-424 sites). How-
ever, the changes associated with S6K1 during forskolin treat-
ment were unaffected by the drug treatment.

Treatment of cardiocytes with U0126 blocked only the TPA-
stimulated but not the insulin- or forskolin-stimulated mTOR
phosphorylation. This suggests that the c-Raf/MEK pathway is
important for the Ser-2448 phosphorylation of mTOR. We also
tested whether U0126 treatment results specifically in the loss
of ERK1/2 activation but not the other members of the MAPK
family such as the p38 MAPK. Pretreatment of cardiocytes
with U0126 strongly blocked the TPA-stimulated ERK1/2 acti-
vation (Fig. 5). However, its blocking effect on the TPA-stimu-
lated p38 MAPK activation was weak. As observed in the
previous experiment (Fig. 2B), only forskolin treatment but not
insulin treatment resulted in p38 MAPK activation. However,
such activation was not significantly altered by the pretreat-
ment with U0126. Overall, these data demonstrate that U0126
treatment, which is known to block the MEK1/2 activation,
results in the loss of TPA-stimulated ERK1/2 activation and
mTOR phosphorylation, and these effects are very similar to
C4B effect in TPA-stimulated cardiocytes. The loss of these
kinase activities is observed with the loss of S6K1 phosphory-
lation and activation, demonstrating that the c-Raf/MEK path-
way is critical for the TPA-mediated S6K1 activation.

Effect of ERK1/2-specific Phosphatase (MKP-3) Expression
on S6K1 Activation—Next, we analyzed the importance of
ERK1/2 as a downstream component of c-Raf/MEK signaling in
mediating S6K1 activation. Because no specific pharmacologi-
cal inhibitors or dominant negative constructs have been de-
veloped for blocking ERK1/2, we used MKP-3, a dual specific
ERK1/2 phosphatase, to dephosphorylate and inactivate
ERK1/2 (53) in an adenoviral construct similar to that for C4B.

Effect of MEK1/2 Inhibitor (U0126) on agonist-stimu-
lated S6K1 activation in adult cardiocytes. Cultured adult cardio-
cytes were preincubated for 30 min in the absence or presence of 10 μM
U0126 and then stimulated for 30 min with 200 nM TPA, 100 nM
insulin, or 1 μM forskolin. Unstimulated cardiocytes served as controls.
Cells were processed as described under "Materials and Methods," and
the samples were used for Western blot analysis using non-selective
and phosphorylation state-specific antibodies. Western blot shows elec-
trophoretic mobility change and phosphorylation status at specific sites
of S6K1 isoforms, mTOR, ERK1/2, and p38 MAPK. The results were
confirmed in two other independent experiments.
The infection of cardiocytes was performed as described under "Materials and Methods." We determined the concentration of adenovirus sufficient to block primarily the TPA-stimulated ERK and S6K1 activation. Infection of cardiocytes with 2 m.o.i. of MKP-3 adenovirus but not β-galactosidase adenovirus lowered the TPA-stimulated ERK1/2 activation, and this effect was observed more significantly at 10 m.o.i. of MKP-3 adenovirus (Fig. 6). This suggests that MKP-3 inactivates ERK1/2, as reported previously (54). Next we analyzed the effect of MKP-3 expression on the agonist-stimulated S6K1 activation. The TPA-stimulated retarded electrophoretic mobility of both S6K1 isoforms was substantially blocked in cardiocytes that were infected with the low concentration of MKP-3 adenovirus (2 m.o.i.) but not in β-galactosidase virus-infected cardiocytes. At the higher concentration of MKP-3 adenovirus (10 m.o.i.) but not β-galactosidase adenovirus, the change in TPA-stimulated electrophoretic mobility is almost completely lost. However, such a change in electrophoretic mobility in insulin- and forskolin-stimulated cardiocytes was not significantly affected by MKP-3 expression. These data suggest that ERK1/2 inactivation has a profound effect on the TPA-stimulated S6K1 activation. Analysis of the phosphorylation status of S6K1 demonstrates that both the Thr-389 phosphorylation and phosphorylation at specific sites of S6K1 isoforms, mTOR, ERK1/2, and p38 MAPK. The results were confirmed in two other independent experiments.

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**Effect of Rapamycin on S6K1 Activation**—Because our data demonstrate that c-Raf/MEK/ERK controls PKC-mediated S6K1 activation, we analyzed the importance of MAPK family members. Both U0126 that blocked the MEK/ERK activation and MKP-3 that blocked ERK1/2 activation, showed a partial effect on the p38 MAPK activation. Therefore, it is possible that either ERK1/2 and/or p38 MAPK contribute to the S6K1 activation. p38 MAPK can be blocked with specific SB compounds, such as SB203580 and SB202190. Pretreatment of cardiocytes with these drugs exhibited the following changes (Fig. 7): (i) no significant changes in the TPA-stimulated S6K1 activation and Thr-421/Ser-424 phosphorylation except a low level drop in the Thr-389 phosphorylation, (ii) insulin-stimulated S6K1 activation (retarded electrophoretic mobility) and Thr-389, Thr-421/Ser-424 phosphorylation were partially blocked, and (iii) forskolin-stimulated S6K1 activation and phosphorylation were unaffected. Therefore, in the case of TPA-stimulated S6K1 activation, the phosphorylation of Thr-421/Ser-424 sites for which p38 MAPK could serve as a potential upstream kinase, is unaffected by the action of SB compounds. Furthermore, although TPA- and insulin-stimulated Thr-389 phosphorylation was partially blocked by SB compounds, mTOR phosphorylation was not affected significantly, especially in the case of TPA- and forskolin-stimulated cardiocytes, indicating mTOR activation alone is not sufficient for the Thr-389 phosphorylation. Overall, these studies performed with SB compounds demonstrate that p38 MAPK does not contribute significantly to S6K1 activation and Thr-421/Ser-424 phosphorylation.

**Effect of Rapamycin on S6K1 Activation**—mTOR is a Ser/Thr kinase and a critical component for S6K1 activation. The functional role of this kinase can be blocked with nanomolar concentrations of rapamycin. Therefore, we analyzed the effect of rapamycin on the phosphorylation pattern of S6K1 during all
S6K1 Activation by c-Raf/MEK/ERK Pathway in Adult Cardiomyocytes

**Fig. 8. Effect of rapamycin on agonist-stimulated S6K1 activation in adult cardiocytes.** Cultured adult cardiocytes were preincubated for 30 min in the absence or presence of 2 nM rapamycin and then stimulated for 30 min with 200 nM TPA, 100 nM insulin, or 1 μM forskolin. Unstimulated cardiocytes served as controls. Samples were used for Western blot analysis using non-selective and phosphorylation state-specific antibodies for S6K1, mTOR, and S6 protein. Western blot shows electrophoretic mobility change and phosphorylation status at specific sites of S6K1 isoforms, mTOR, and S6 protein. The level of S6 protein is also determined by analyzing with a non-selective anti-S6 protein antibody. The results were confirmed in two other independent experiments.

Three types of stimulants. The activation of both S6K1 isoforms by all three stimulants was blocked when cardiocytes were pretreated with rapamycin (Fig. 8). Furthermore, rapamycin treatment blocked Thr-389 phosphorylation, indicating that the phosphorylation at this site requires mTOR activity. Interestingly, rapamycin pretreatment did not affect the TPA-stimulated Thr-421/Ser-424 phosphorylation, although there were changes in the migration of such phosphorylated S6K1 species during SDS-PAGE separation. The changes in the position of Thr-421/Ser-424 phosphorylated protein bands upon rapamycin pretreatment are likely due to faster electrophoretic mobility of S6K1 following the loss of phosphorylation at other potential sites. In the case of insulin-treated cardiocytes, this phosphorylation, which was relatively lower when compared with the TPA-treated cardiocytes, was brought to the basal level by the rapamycin pretreatment. Taken together, these data demonstrate that (i) the Thr-389 phosphorylation induced by all three agents can be blocked with rapamycin and (ii) the phosphorylation at Thr-421/Ser-424 sites of S6K1 in TPA-treated cardiocytes proceeds independent of mTOR.

Rapamycin treatment did not block the phosphorylation of mTOR at the Ser-2448 site significantly, indicating that the drug affects only the activity of mTOR but not the upstream kinase that is responsible for the mTOR phosphorylation at the Ser-2448 site.

**DISCUSSION**

Ribosomal biogenesis is an important cellular event for mediating hypertrophic cardiac growth (1–5), and the activation of S6K1 is critical for the augmented ribosomal biosynthesis (9–12, 55). S6K1 phosphorylates ribosomal protein S6, which is a key component in the translation of subset mRNA transcripts that possesses a tract of pyrimidines (5'-TOP mRNAs) in the 5'-untranslated region (5'-UTR) (6, 7, 23). 5'-TOP mRNAs generally encode ribosomal proteins and elongation factors, and therefore, the overall translational capacity of cells increases substantially following S6K1 activation. In 48-h pressure-overloaded myocardium, accelerated protein synthesis can be observed with an enhanced new steady-state level (43). Therefore, initial S6K1 activation and ribosomal biogenesis are major myocardial cellular events for sustained hypertrophic growth. The importance of S6K1 activation during pressure overload hypertrophy has been shown recently using angiotensin type II-receptor knockout mice (56). These mice were found to have reduced levels of p70S6K and an absence of pressure overload-induced hypertrophic cardiac growth. The link between S6K1 activation and cell growth has been well documented in other cell types using rapamycin (10, 11, 25, 57) and through microinjection of neutralizing antibodies into cells (15, 17) both of which selectively suppressed S6K1 activation and impeded cell growth. S6K1 activation has been demonstrated in hypertrophying myocardium (42, 58) and in cardiocytes stimulated with hypertrophic agents (39, 59–61). However, the major signaling pathway for S6K1 activation and its importance in the context of hypertrophic growth has not been studied in detail.

The S6K1 activation process, which relies on the sequential interplay between multiple phosphorylation sites and signal transduction pathways (62–64), is complex and not completely understood. Several independent signaling pathways have been identified for S6K1 activation depending upon cell types and the nature of the stimulants (30–36), and these pathways are activated subsequent to at least three independent agonists’ stimulation: (i) TPA that activates a PKC-dependent pathway, (ii) insulin that activates a PI3K-dependent pathway, and (iii) forskolin that activates a protein kinase A (PKA)-dependent pathway. Activation of all these pathways has been demonstrated in pressure-overloaded myocardium by several research groups (58, 61, 65–67), although our earlier study (42) demonstrated that the S6K1 activation was accompanied by the activation of PKC but not PI3K pathway in 1- to 4-h pressure overload myocardium. Therefore, we used cultured adult feline cardiocytes to explore the importance of potential downstream players of PKC, namely, c-Raf, MEK, and MAPK family members for S6K1 activation.

Our study demonstrates that stimulation of adult cardiocytes with TPA results in S6K1 activation, which is accompanied by the activation of c-Raf as well as the MAPK family members, ERK1/2 and p38 MAPK, but not JNK. It has been well established that the c-Raf-mediated ERK1/2 activation occurs via the activation of MEK1/2, and our studies with U0126 confirm that this kinase is active in TPA-stimulated cardiocytes. However, stimulation with insulin and forskolin, although they phosphorylate and activate S6K1 and mTOR, did not result in the activation of c-Raf and ERK1/2. Therefore, the contribution of the c-Raf/MEK/ERK pathway to S6K1 activation can be expected mostly during TPA stimulation but not with the other two stimulants.

In the case of S6K1 phosphorylation, although Thr-389 phosphorylation was observed during all three types of stimulations, the extent of Thr-421/Ser-424 phosphorylation was stronger in TPA-stimulated cardiocytes. Furthermore, in TPA-stimulated cardiocytes, the Thr-421/Ser-424 phosphorylation occurs prior to the Thr-389 phosphorylation, indicating the possibility that the pseudosubstrate phosphorylation occurs first to facilitate the phosphorylation of Thr-389, as suggested previously (68). However, in forskolin-stimulated cardiocytes,
the basal level phosphorylation at the Thr-421/Ser-424 sites appears to be sufficient for the Thr-389 phosphorylation. Both PKA and PI3K activation have been demonstrated in pressure-overloaded myocardium (58, 61, 67), indicating the possibility that they contribute to S6K1 activation. However, our findings suggest that PKC-mediated signaling contributes significantly to the S6K1 activation for the following reasons: (i) only in TPA-, but not insulin- or forskolin-treated cardiocytes, the Thr-421/Ser-424 phosphorylation occurs similar to that with pressure overload, (ii) our previous study (42) demonstrates that S6K1 activation in 1- to 4-h pressure-overloaded feline myocardium was not accompanied by PI3K activation, (iii) similar to pressure-overloaded myocardium, TPA-stimulated cardiocytes demonstrate S6K1 activation and phosphorylation, accompanied by the phosphorylation/activation of c-Raf and MAPK (ERK1/2 and p38 MAPK), and (iv) analysis of PKB, a downstream kinase of PI3K, in 1-h pressure-overloaded RV did not show appreciable change in its phosphorylation level when compared with the unloaded control LV (data not shown). Therefore, a major focus of this study was to determine the key intermediary players responsible for the PKC-mediated S6K1 activation in adult feline cardiomyocytes.

Whereas previous studies have shown that Raf/MAPK signaling is neither necessary nor sufficient for S6K1 activation by mitogens (34, 69, 70), our data support recent studies demonstrating that the expression of either active c-Raf or MEK is sufficient for S6K1 activation in an ERK-dependent manner (29, 37, 71). Furthermore, MEK has been shown to activate a closely related S6K1 member, S6K2, in adult rat cardiocytes (39), although the importance of this isoform has not been studied in detail in pressure-overloaded myocardium. MEK1/2, but not ERK1/2, has been shown to be important for S6K1 activation in response to insulin or phorbol esters in adipocytes (72, 73). Therefore, in vivo activation of either c-Raf or MEK alone appears to be sufficient for S6K1 activation, although the importance of ERK1/2 in this process is not clear.

To explore the possibility that the c-Raf/MEK/MAPK pathway contributes to the PKC-mediated S6K1 activation, we used the dominant negative approach to block c-Raf, the pharmacological agents to block the MEK, and the overexpression of a specific phosphatase to block ERK. Expression of C4B, resulting in dominant negative c-Raf concentrations (~20-fold higher than the endogenous levels, blocked the TPA-stimulated activation of c-Raf, ERK1/2, and p38 MAPK. Whereas C4B expression abolished the TPA-stimulated phosphorylation of S6K1 and S6 protein, it does not exhibit any such effect in the insulin- and forskolin-stimulated S6K1 activation and S6 protein phosphorylation. This observation suggests that: (i) the insulin- and forskolin-stimulated S6K1 activation, which is known to occur via PI3K and PKA activation, respectively, is independent of the PKC-stimulated pathway that requires c-Raf, and (ii) adenoviral expression of C4B specifically blocks the c-Raf-mediated effect and does not have nonspecific effects on other signaling pathways. Taken together, these studies indicate that c-Raf is a specific downstream player of PKC during TPA-stimulated S6K1 activation.

Similar to the C4B experiment, U0126 treatment blocks the TPA-induced phosphorylation of S6K1 and mTOR. This clearly suggests that MEK1/2 is a critical intermediary of PKC-mediated S6K1 activation. In the case of insulin- and forskolin-stimulated S6K1 activation, U0126 treatment, unlike the C4B expression, caused a partial blocking of S6K1 phosphorylation. Therefore, although MEK1/2 is an important downstream effector of c-Raf, other pathways that mediate S6K1 activation seem to rely on the basal activity of MEK1/2. In this context, the basal MEK1/2 activity has been shown to be important for insulin (72, 73)- and epidermal growth factor (71)-stimulated S6K1 activation. Overall, our study demonstrates that activation of MEK1/2 alone can be sufficient for the mTOR and S6K1 phosphorylation at specific sites in adult cardiocytes, suggesting that MEK1/2 is a critical player functioning downstream of c-Raf during PKC-mediated S6K1 activation. In support of this, previous studies demonstrate that c-Raf is sufficient to activate S6K1 in CCL39 cells (37) and that MEK1/2 is important for the S6K1 activation in HEK293E cells (71). In adult cardiocytes, a recent study also demonstrates that MEK1/2 is important for the activation of S6K2, a structurally related kinase (39).

To identify downstream components of MEK1/2 signaling, we focused on MAPK. Our studies using MKP-3 adenovirus suggest that ERK1/2 is a potential downstream player for the PKC/c-Raf/MEK pathway leading to S6K1 activation. The TPA-stimulated changes, including the retarded electrophoretic mobility and phosphorylation at Thr-421/Ser-424 sites of S6K1, are blocked significantly in cells expressing MKP-3. However, in insulin- and forskolin-stimulated cells, the retarded electrophoretic mobility is not significantly affected. Analysis of the phosphorylation pattern of S6K1 demonstrates that MKP-3 significantly blocks both the Thr-421/Ser-424 and the Thr-389 phosphorylations when these cells are stimulated with TPA. There are at least two possibilities for the loss of Thr-389 phosphorylation during MKP-3 expression: (i) both the basal, as observed during forskolin stimulation, and the stimulated, as observed during TPA stimulation, levels of Thr-421/Ser-424 phosphorylation, which is critical for the subsequent Thr-389 phosphorylation, are abolished and/or (ii) the phosphorylation and activation of other potential intermediates, such as mTOR that controls the Thr-389 phosphorylation, is affected. Our data on the Ser-2448 phosphorylation of mTOR indicate that the TPA- but not insulin- or forskolin-stimulated phosphorylation is lost in MKP-3-expressing cells. This suggests that the mTOR phosphorylation at Ser-2448 site is mediated specifically via c-Raf/MEK/ERK pathway in TPA-stimulated cardiocytes. Taken together these data indicate that the TPA-stimulated phosphorylation of both S6K1 at Thr-421/Ser-424 and Ser-389 sites and mTOR at Ser-2448 site requires the c-Raf/MEK/ERK pathway and that this pathway does not appear to be critical during insulin and forskolin stimulation. In support of our observation, a recent study (66) demonstrates that ERK1/2 is complexed with p70S6K, suggesting the possibility that ERK1/2 is a direct S6K1 kinase for the phosphorylation of one or more Ser/Thr-Pro sites, such as the Thr-421/Ser-424 sites in the pseudosubstrate domain.

It is important to note that the MKP-3 expression, compared with the C4B expression, was performed using significantly lower concentration of adenovirus (2–10 versus 250 m.o.i., respectively). Although the same cytomegalovirus promoter drove both viral expressions, the cellular concentrations of such expressed proteins depend upon their turnover rates. Furthermore, their biological effect can vary depending upon their specific activity. Although MKP-3 has been shown to be a phosphatase specific to ERK1/2, a high level expression can result in nonspecific effects on other phosphorylated proteins, including S6K1. Therefore, we used appropriate viral concentrations to demonstrate the specific effects of the expressed proteins on the TPA-stimulated S6K1 activation relative to the insulin- and forskolin-stimulated S6K1 activation. Thus by infecting cardiomyocytes at a relatively lower concentration of MKP-3 adenovirus (2 and 10 m.o.i.), we were able to demonstrate that the MKP-3 significantly blocked the TPA- but not the insulin- or forskolin-stimulated S6K1 activation. On the other hand, the dominant negative c-Raf (C4B) virus, at a relatively higher concentration (250 m.o.i.), also blocked spe-
cifically the TPA-stimulated S6K1 activation. These studies demonstrate that c-Raf is a major downstream player of the PKC-mediated S6K1 activation and that ERK1/2, which functions further down stream c-Raf, is also a critical player to the TPA-stimulated S6K1 activation, although ERK1/2, unlike c-Raf, may also contribute partially to the forskolin-stimulated S6K1 activation.

Our experiments using two types of SB compounds demonstrate that the S6K1 activation by all three stimulators is not affected appreciably during this drug treatment. Importantly, SB compounds did not block the TPA-stimulated Thr-421/Ser-424 phosphorylation and retarded electrophoretic mobility of S6K1. Furthermore, these compounds did not block mTOR phosphorylation at Ser-2448, although the reason for the partial loss of Thr-389 phosphorylation during all three types of stimulation is not clear at the present time. Therefore, although both ERK1/2 and p38 MAPK activity are sharply reduced in Mkp-3-expressing cells, the loss of S6K1 activation and Thr-421/Ser-424 phosphorylation in these cells is mostly due to the loss of ERK1/2 activity. A similar study (37) performed using Mkp-1 demonstrates that the loss of ERK1/2 activity does not affect S6K1 activation. It is not clear whether the differences are due to a spatial difference in the loss of ERK1/2 activity, because Mkp-1 is a nuclear enzyme. Overall, our studies strongly indicate that ERK1/2 is a major downstream kinase, transferring the c-Raf/MEK signal to S6K1 activation. However, our studies do not rule out the possibility that one or more kinases necessary for S6K1 activation are regulated directly by c-Raf and/or MEK.

Studies using transgenic mice demonstrate that PKCε, compared with other isoforms, contributes to hypertrophic growth in a significant manner (74). In pressure-overloaded myocardium, our earlier study (42) shows the activation of at least three PKC isoforms, which includes the PKCε isoform. Furthermore, stimulation of cardiomyocytes with hypertrophic agents, such as phynephrine, results in the membrane localization of PKCe and contributes to the activation of the c-Raf/MEK/ERK pathway (75). A latent complex between PKCe, c-Raf, and Ras has been identified recently in other cell types (76). Based on these reports and our present findings, we propose the following model to describe our studies. We propose that the activation of a specific PKC isoform contributes significantly to the S6K1 activation in pressure-overloaded myocardium. c-Raf is the major effector molecule that connects the PKC signaling to MEK1/2 for the activation of S6K1. MEK1/2, via regulating ERK1/2, controls both the Thr-421/Ser-424 phosphorylation of S6K1 as well as mTOR phosphorylation required for the Thr-389 phosphorylation of S6K1. All these phosphorylations are important for subsequent Thr-229 phosphorylation and kinase activation, as described previously (24).

Overall, we demonstrate that c-Raf and its downstream components, MEK1/2 and ERK1/2, contribute in a significant way to the PKC- but not to the PI3K or PKA-mediated S6K1 activation in adult feline cardiomyocytes. Because we observe activation of the PKC/c-Raf/ERK pathway in pressure-overloaded myocardium, this pathway might be critical for hypertrophic cardiac growth via S6K1 activation and ribosomal biogenesis.

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c-Raf/MEK/ERK Pathway Controls Protein Kinase C-mediated p70S6K Activation in Adult Cardiac Muscle Cells
Yoshihiro Iijima, Martin Laser, Hirokazu Shiraishi, Christopher D. Willey, Balasubramanian Sundaravadiel, Lin Xu, Paul J. McDermott and Dhandapani Kuppuswamy

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