Protein Kinase C and Protein Kinase A Inhibit Calcium-dependent but not Stress-dependent c-Jun N-terminal Kinase Activation in Rat Liver Epithelial Cells

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Xiong Li‡, Hong Yu§, Lee M. Graves §§, and H. Shelton Earp §§§

From the Departments of ‡Pharmacology and §Medicine, §§University of North Carolina Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

In rat liver epithelial cells (GN4), angiotensin II (Ang II) and thapsigargin stimulate a novel calcium-dependent tyrosine kinase (CADTK) also known as PYK2, CAKβ, or RAFTK. Activation of CADTK by a thapsigargin-dependent increase in intracellular calcium failed to stimulate the extracellular signal-regulated protein kinase pathway but was well correlated with a 30–50-fold activation of c-Jun N-terminal kinase (JNK). In contrast, Ang II, which increased both protein kinase C (PKC) activity and intracellular calcium, stimulated extracellular signal-regulated protein kinase but produced a smaller, less sustained, JNK activation than thapsigargin. 12-O-Tetradecanoylphorbol 13-acetate (TPA), which slowly activated CADTK, did not stimulate JNK. These findings suggest either that CADTK is not involved in JNK activation or PKC activation inhibits the CADTK to JNK pathway. A 1-min TPA pretreatment of GN4 cells inhibited thapsigargin-dependent JNK activation by 80–90%. In contrast, TPA did not inhibit the >50-fold JNK activation effected by anisomycin or UV. The consequence of PKC-dependent JNK inhibition was reflected in c-Jun and c-Fos mRNA induction following treatment with thapsigargin and Ang II. Thapsigargin, which only minimally induced c-Fos, produced a much greater and more prolonged c-Jun response than Ang II. Elevation of another intracellular second messenger, cAMP, for 3–15 min also inhibited calcium-dependent JNK activation by ~80–90% but likewise had no effect on the stress-dependent JNK pathway. In summary, two pathways stimulate JNK in cells expressing CADTK, a calcium-dependent pathway modifiable by PKC and cAMP-dependent protein kinase and a stress-activated pathway independent of CADTK, PKC, and cAMP-dependent protein kinase; the inhibition by PKC can ultimately alter gene expression initiated by a calcium signal.

Mitogen-activated protein kinases (MAPKs)¹ are important intermediates in signaling pathways that transduce extracellular signals into intracellular responses and have been implicated in a wide array of physiological processes including cell growth, differentiation, and apoptosis. There are at least three and perhaps four subfamilies of MAPKs: (i) p44 and p42 MAPKs (MAPK1 and -2) also referred to as extracellular signal-regulated protein kinases 1 and 2 (ERK1 and -2) (1); (ii) p54 and p46 stress-activated protein kinases 1 and 2, also referred to as the c-Jun N-terminal kinase 1 and 2 (JNK1 and -2) (2); (iii) p38 MAPK (3), the closest mammalian homologue of the yeast osmosensing ERK HOG1; and (iv) a potential subfamily member, the as yet unsequenced, 88-kDa c-Fos-regulating protein kinase (4). The known subfamilies are related by three common characteristics. First, they share high sequence homology and presumably similar structure and conformation (5). Second, all appear to be activated by protein kinase cascades initiated by small guanine nucleotide-binding proteins in the Ras superfamily with subsequent mitogen-activated protein kinase kinase kinases (MEKKs) activation (6, 7). Third, the last step in activation requires a dual specificity kinase, e.g. the mitogen-activated protein kinase kinases (8) and stress-activated protein kinase kinases (2, 7), which phosphorylate both Thr and Tyr residues. Dual site phosphorylation of the sequence TGY, TPY, or TGY at the activators of the catalytic clefs of these protein kinases is the defining property of ERK, JNK, and p38 MAPK, respectively (9).

On the other hand, each subfamily also has distinct characteristics (7). First, they are activated by different stimuli, ERKs by growth factor receptor tyrosine kinases (6) or PKC (5) and JNK by stress signals (2), such as inflammatory cytokines or UV irradiation. Second, each has substrate preferences (10), e.g. Elk-1, SAP1, and phospholipase A2 for ERKs, c-Jun and ATF2 for JNK, and ATF2 and Max for p38 MAPK. Third, activation regulates distinct cellular responses; ERKs lead to cell growth and differentiation (6), while in some cells JNKs and p38 MAPK inhibit cell growth or may promote either necrotic or apoptotic cell death (2).

The present study examines the calcium-dependent pathway to JNK activation (11) and defines two mechanisms (PKC- and PKA-dependent) that modify its output. We had previously shown that the calcium-dependent JNK pathway was PKC-independent as demonstrated by the fact that Ang II stimulates JNK more effectively in PKC-depleted cells than in control cells (11). This phenomenon was initially thought to be secondary to abrogation of the PKC-dependent negative feedback on the Ang II inositol 1,4,5-trisphosphate/calcium signal (12). The stimulation of JNK by Ang II or thapsigargin appears to be initiated by a nonreceptor calcium-dependent tyrosine kinase kinase; EGF, epidermal growth factor; 8-CPT-cAMP, 8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic monophosphate sodium salt.
kinase (CADTK) that we have purified and cloned (13, 14). CADTK is highly related to the focal adhesion tyrosine kinase, p125Fak, and is the same tyrosine kinase identified recently by three other groups (PYK2, CAKβ, and RAFTK) (15–17). Thapsigargin-dependent activation of JNK is well correlated with CADTK expression and activation (14), and Tokiwa et al. have provided direct evidence for the CADTK/PYK2 to JNK pathway (18). However, the role of CADTK in JNK activation was thrown into doubt when we established that tritiated phosphatidylcholine 13-acetate (TPA)-dependent activation of PKC stimulated CADTK but not JNK. We therefore investigated whether PKC activation had a second role, the inhibition of the CADTK signal to JNK. Our results showed that prior stimulation of PKC decreased calcium-dependent JNK activation by ~80–90%; inhibition was observed with TPA treatment either before or shortly after the addition of thapsigargin to cultured cells. Further studies showed that this inhibitory TPA action was mediated by PKC and not by the TPA-dependent secondary activation of ERK. Another second messenger pathway, PKA, also inhibited calcium-dependent JNK activation. However, neither PKC nor PKA blunted anisomycin-dependent JNK activation, demonstrating the existence of at least two independent pathways to JNK (calcium and stress) in cells expressing CADTK. The PKC-dependent attenuation of JNK activation has biological consequences, e.g. thapsigargin, which produces a calcium signal, is a much stronger inducer of c-Jun mRNA than Ang II, which generates both calcium and PKC signals. In summary, cells expressing CADTK may use PKC or PKA to balance ERK- or JNK-dependent alteration in gene expression in response to G-protein-coupled receptors.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant EGF was purchased from Life Technologies, Inc. Angiotensin II (Ang II) was purchased from Sigma and prepared in 50 mM acetic acid as stock solution. TPA, thapsigargin, and bis(2-o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl) ester were purchased from Sigma and Biomol, respectively, and prepared as stock solutions in dimethyl sulfoxide (Me2SO). S-CPT-cAMP was purchased from Biomol. Anti-Tyr(P) monoclonal antibodies R20H and pT66 were purchased from Transduction Laboratories and Sigma, respectively. Anti-ERK polyclonal antibody k-23 was purchased from Santa Cruz Biotechnology, and anti-CADTK polyclonal antibody was produced by using GST-CADTK fusion protein as described previously (14).

Cell Culture and Cell Lysate Preparation—Rat liver epithelial cells, GN4, were grown in Richter's improved minimal essential medium with 0.1% insulin supplemented with 10% fetal bovine serum as described previously (13). Cell lysate preparation was performed essentially as described previously (13). Briefly, cells treated with agonists were homogenized in ice-cold cell lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 50 mM NaF, and 10% (v/v) glycerol) with freshly added 1 mM Na3VO4, 20 mM p-nitrophenyl phosphatase, 0.1 mM Na3VO4, 2 mM dithiothreitol). The pellets were resuspended in 35 µg of cell lysates were incubated with 12 µl of GST-c-Jun (1–79) linked to Sepharose beads as the substrate (11). Briefly, 50 µg of cell lysates were incubated with 12 µl of GST-c-Jun beads in lysis buffer. The reaction mixture was then rotated for 2 h. After the incubation, the reaction mixture was pelleted by centrifugation and washed three times with lysis buffer and once with kinase buffer (20 mM HEPES (pH 7.6), 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 0.1 mM Na3VO4, 2 mM dithiothreitol). The pellets were resuspended in 35 µl of kinase buffer containing 0.5 µCi of [γ-32P]ATP. Samples were incubated for 10 min at 30 °C and chilled to stop the kinase reaction. After removing excess kinase buffer, samples were boiled with 10 µl of 3 × SDS-PAGE sample buffer and subjected to 12% SDS-PAGE, followed by Coomassie Blue staining, autoradiography, and PhosphorImager analysis.

ERK Activation Assays—Activation of ERKs was determined as described previously (14). Briefly, 15 µg of cell lysates were resolved by 15% low bisacrylamide SDS-PAGE. Proteins were then transferred to nitrocellulose membranes for analysis by immunoblotting with the anti-ERK antibody, K-23. The immunoblot was incubated with goat anti-rabbit horseradish peroxidase-conjugated antibodies and developed according to the manufacturer's procedure (Amersham Corp.).

Anti-Tyr(P), CADTK Immunoprecipitation, and Immunoblotting—In a typical experiment, 500 µg of cell lysates were immunoprecipitated by incubation with the antibody for 2 h at 4 °C, and then 20 µl of protein A or protein G-agarose beads (Santa Cruz) were added for an additional 1 h. Immune complexes were collected by centrifugation, washed three times with lysis buffer, and then resuspended in SDS-PAGE sample buffer. Samples were subjected to SDS-PAGE and transferred to Immobilon (Millipore Corp.). Proteins were detected by incubating the blots with the appropriate antibody and visualizing by using ECL reagents as described before (14).

Northern Blotting—Total RNA of GN4 cells, treated with Ang II (1 µM), EGF (100 ng/ml), and thapsigargin (2 µM) for the indicated times, were prepared with Trizol® solution according to the manufacturer's instructions (Life Technologies, Inc.). 30 µg of total RNAs were separated on 1.2% formaldehyde agarose gel and transferred to Zeta-Prob® GT blotting membrane according to the manufacturer's instructions (Bio-Rad). The membrane was probed with cDNA probes labeled with [α-32P]dCTP by the random primed DNA labeling method (Boehringer Mannheim), washed with washing buffer according to the manufacturer's instructions, and then subjected to autoradiography. 28S and 18S ribosomal RNA were stained by 1% methylene blue to show even loading.

RESULTS

Protein Kinase C Inhibits Calcium-dependent JNK Activation—Previous attempts to dissect the role of calcium and PKC in GN4 cell signaling revealed that both Ang II and thapsigargin increased tyrosine phosphorylation as well as JNK and AP-1 activity in GN4 cells depleted of >95% of their PKC (11, 19, 20). Moreover, Ang II-dependent JNK activation was at least 2-fold greater in PKC-depleted cells, a fact that we initially ascribed to the negative feedback of PKC on Ang II signaling (12). When we identified CADTK as the major calcium-dependent tyrosine kinase in GN4 cells (13) and correlated its stimulation and expression with JNK activation (14), a linkage between CADTK and JNK activation was apparent with one exception. TPA treatment of GN4 cells activated CADTK but not JNK. Either CADTK was not causally related to JNK activation or TPA inhibited JNK activation at a site distal to CADTK.

To elucidate an inhibitory role for PKC, we first examined in detail the time course of calcium-dependent JNK activation by thapsigargin and Ang II. We had previously demonstrated that Ang II was a stronger activator of JNK than thapsigargin in cells depleted of >95% of their PKC by an overnight TPA pretreatment (11). In the present studies, we used unpretreated GN4 cells and showed the opposite result; thapsigargin stimulated JNK activity to a greater extent and for a longer duration than Ang II (Fig. 1). The initial burst of JNK activity was similar for both agonists, but by 20–30 min the magnitude of the thapsigargin response was consistently 2–2.5-fold greater in repeated experiments.

To directly test the hypothesis that the PKC signal inhibited the calcium-dependent JNK pathway, we pretreated GN4 cells with 100 nM TPA for 10 min. Pretreatment for as little as 1 min decreased thapsigargin-dependent JNK activation by 80–90% (Fig. 2A), and a 15-s pretreatment showed a significant inhibition (Fig. 3A). In contrast, TPA pretreatment did not alter the stress pathway typified by anisomycin-dependent JNK activation (Fig. 2A) or exposure to 100 J/m2 of UV (data not shown). These results delineated separate JNK activation pathways, one inhibited by TPA, the other not. Interestingly, TPA added for 1 or 2.5 min after thapsigargin also decreased calcium-dependent JNK activation by ~50% at 30 min, mimicking the difference observed between treatment with thapsigargin (calcium) and Ang II (calcium and PKC) at 30 min (Fig. 1). TPA treatment 5 min after thapsigargin had little or no
inhibitory effect, suggesting that there is a limited time span in which the PKC signal can influence the CADTK to JNK pathway (Fig. 2B). A dose response performed using a 10-min TPA preincubation showed that as little as 10 nM TPA blocked thapsigargin-dependent JNK activation (Fig. 3B). This effect of TPA was mediated by its ability to activate PKC; the TPA inhibitory effect was abolished by depleting cells of 95% PKC using an overnight TPA (5 μM, 18 h) pretreatment (data not shown). Last, because the PKC signal produced by TPA activation stimulates ERK, we tested whether it was the PKC or the ERKs activation attenuating the CADTK to JNK pathway. EGF strongly activates ERKs in GN4 cells, but pretreatment with EGF for 1–5 min failed to inhibit thapsigargin-dependent JNK activation (Fig. 3C). This suggests that inhibition of the CADTK to JNK pathway is mediated directly by a TPA-sensitive PKC isoform.

To test whether TPA simply blocked the putative first step of Ang II or thapsigargin action, the activation of CADTK, GN4 cells were pretreated with or without TPA, and CADTK tyrosine phosphorylation was examined (Fig. 4). TPA pretreatment did not inhibit, and in fact enhanced, thapsigargin-dependent CADTK tyrosine phosphorylation. In addition, TPA also increases CADTK tyrosine kinase activity assessed by immune complex kinase activity (14) using poly(Glu4Tyr) as a substrate (data not shown). Therefore, PKC must interfere with elements downstream of CADTK in the calcium-dependent JNK activation pathway.

**Thapsigargin Stimulates Prolonged JNK Activation and c-Jun Expression**—To test the biological consequences of the

![FIGURE 1](image1.png)

**FIG. 1.** Thapsigargin stimulation resulted in a sustained JNK activation. Rat liver epithelial cells (GN4) were treated with Ang II (1 μM) or thapsigargin (2 μM) for the indicated time points. Cell lysates were prepared and incubated with 12 μl of GST-c-Jun-(1–79) beads. JNK activity assay was performed as described under "Experimental Procedures." The phosphorylated product was subjected to SDS-PAGE and analyzed by autoradiography, and the fold activation was determined by PhosphorImager analysis. These data and those shown in Figs. 2–4 and Figs. 6–8 are representative of at least three experiments.

![FIGURE 2](image2.png)

**FIG. 2.** TPA pretreatment inhibited calcium-dependent, but not anisomycin-dependent JNK activation. GN4 cells were pretreated with TPA (100 nM) for the indicated time points, before or after a 30-min treatment with thapsigargin (2 μM) or anisomycin (100 ng/ml). Cell lysates were prepared, and JNK activity assays were performed. A, TPA pretreatment inhibited JNK activation by thapsigargin but did not inhibit JNK activation by anisomycin. B, TPA at the indicated times after thapsigargin (Posttreatment) also inhibited JNK activation by calcium if TPA was added 1 or 2.5 min after thapsigargin. C, TPA treatment did not stimulate JNK activity.
PKC and PKA Inhibit Calcium-dependent JNK Activation

In some cell types, increased intra-
cellular cAMP and PKA activity inhibits ERK (21–23) or JNK activation (24–26). We investigated whether increasing cAMP
would inhibit calcium-dependent JNK activation. GN4 cells were
pretreated with forskolin/IBMX or 8-CPT-cAMP after the addition of thapsigargin had little or no effect on JNK activation. These data suggest that forskolin/IBMX and 8-CPT-
cAMP are effective if added prior to the calcium signal and are most effective when provided 5–15 min prior to the calcium
signal. This may indicate that cAMP has a different site of
inhibition than TPA, which inhibits the CADTK to JNK signal
maximally with a 30–60-s preincubation and can inhibit even when added 1–2 min after administering thapsigargin. How-
ever, it is possible that PKA and PKC may share the same site
of inhibition with the PKA signal taking slightly longer to
develop.

We also tested the effect of cAMP on ERK activation. Eleva-
tion of cAMP in GN4 cells had no effect on Ang II-dependent
ERK activation (Fig. 6B). This is different from the action of
cAMP in other cell types (21–23). Since the effect of Ang II on
ERK in GN4 cells is primarily a Ras/Raf-independent process,2
the lack of cAMP inhibition may not be comparable with other
Ras-dependent ERK activation systems. To study the selectiv-
ity of cAMP inhibition for the calcium-dependent JNK activa-
tion, we pretreated cells with forskolin/IBMX followed by Ang
II, thapsigargin, or anisomycin treatment. Like TPA, cAMP
inhibited thapsigargin and Ang II-dependent JNK activation
but failed to affect JNK activation by anisomycin (Fig. 7),
providing further evidence of distinct pathways to JNK stimu-
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lated by calcium/PKC signaling cross-talk, we investigated whether
Ang II, EGF, and thapsigargin treatment differentially affected
Jun and Fos family gene expression. In GN4 cells, the calcium
and PKC signals produced by Ang II activate both JNK and
and PKC predominately activates ERK and has little
effect on JNK, whereas thapsigargin strongly activates JNK
(Fig. 1) and has little effect on ERK activation (11, 14). GN4
cells were stimulated with Ang II, EGF, and thapsigargin for
the indicated times, and total RNA was isolated. Northern blot
analysis (Fig. 5) revealed that thapsigargin strongly stimulated
c-Jun expression with expression increasing for up to 2 h;
thapsigargin had little effect on c-Fos expression. Ang II also
stimulated c-Jun expression, but the induction was weaker and
markedly truncated compared with thapsigargin. Ang II sig-
nificantly stimulated c-Fos expression, consistent with the ef-
ficacy of inhibition for the calcium-dependent JNK activa-
tion. These data support the hypothesis that the negative effect of PKC on Ang II-dependent JNK
activation has important consequences for c-Jun expression.

As expected, EGF strongly activated c-Fos expression. Inter-
estingly, EGF, which had little stimulatory effect on JNK in
GN4 cells (Figs. 3C and 7) (11), rapidly and potently stimulated
c-Jun expression. In contrast to thapsigargin, EGF-dependent
c-Jun expression reached its peak after about 30 min and then
gradually decreased to its basal level.

cAMP Inhibits the Calcium-dependent but Not Stress-
dependent JNK Pathway—In some cell types, increased intra-
acellular cAMP and PKA activity inhibits ERK (21–23) or JNK
activation (24–26). We investigated whether increasing cAMP
would inhibit calcium-dependent JNK activation. GN4 cells were
briefly pretreated with forskolin/IBMX or 8-CPT-cAMP. As
shown in Fig. 6, these compounds have little or no effect on
JNK activation. However, pretreatment with forskolin/IBMX
or cAMP for even 1 min inhibited the subsequent thapsigargin-
dependent JNK activation (Fig. 6A). Longer pretreatment
(5–15 min) inhibited thapsigargin-dependent JNK activation
by ~80–90%. Treatment with forskolin/IBMX or 8-CPT-cAMP
was prepared from GN4 cells treated with Ang II (1 μM), EGF (100
ng/ml), or thapsigargin (2 μM) for the indicated times. 30 μg of total
RNA was separated on a 1.2% formaldehyde agarose gel and probed with radiolabeled c-Jun and c-Fos cDNAs. Samples were stained with methylene blue to assess equal loading of 28 and 18 S RNA.

Fig. 5. Thapsigargin (Thaps) stimulated sustained c-Jun expression but only minimally induced c-Fos expression. Total RNA was prepared from GN4 cells treated with Ang II (1 μM), EGF (100 ng/ml), or thapsigargin (2 μM) for the indicated times. 30 μg of total RNA was separated on a 1.2% formaldehyde agarose gel and probed with radiolabeled c-Jun and c-Fos cDNAs. Samples were stained with methylene blue to assess equal loading of 28 and 18 S RNA.

Fig. 6. Elevation of intracellular cAMP with forskolin/IBMX or 8-CPT-cAMP treatment inhibited calcium-dependent JNK activation, but not Ang II-dependent ERK activation. GN4 cells were pretreated with or without forskolin (25 μM) or forskolin/IBMX (25 and 250 μM, respectively; 15 min) or 8-CPT-cAMP (500 μM, 15 min). These cells were then treated with or without thapsigargin (Thaps; 2 μM) (A) and Ang II (1 μM) (B) for 30 min. Cell lysates were prepared, and JNK activity assays were performed (A) or cell lysates were subjected to 15%
SDS-PAGE for anti-ERK (K-23) immunoblot (B).
determine whether phosphatidylinositol-3 kinase or rapamycin-sensitive kinase were involved in calcium-dependent JNK activation, GN4 cells were pretreated with rapamycin (10 mM) and wortmannin (50 mM, 10 min), doses that totally inhibit thapsigargin-dependent p70S6K activation (27). Neither wortmannin (data not shown) nor rapamycin (Fig. 7) had any effect on calcium-dependent JNK activation. Thus, the calcium-dependent JNK pathway diverges from the calcium-dependent p70S6K pathway; rapamycin and wortmannin only inhibit the latter.

To formally rule out an upstream effect of forskolin/IBMX, rapamycin, or wortmannin on CADTK activation, these compounds were preincubated with GN4 cells before the addition of Ang II. As shown in Fig. 8, neither wortmannin, rapamycin, nor forskolin/IBMX altered Ang II-stimulated CADTK tyrosine phosphorylation. Thus, in GN4 cells, the phosphatidylinositol-3 kinase kinase and/or rapamycin-sensitive kinase may be downstream of a CADTK signal linked to p70S6K activation, but these enzymes are not involved in the CADTK to JNK pathway or the Ang II to CADTK pathway.

**DISCUSSION**

Recent studies using cells other than fibroblasts, e.g. cells of epithelial and hematopoietic origin, increasingly demonstrate a role for G-protein-coupled receptors and/or rises in intracellular calcium as mediators of growth control pathways (30–32) and gene expression (33, 34). Although the role of CADTK in proliferation has not yet been defined, CADTK is expressed in tissue and cell types that respond to G-protein-coupled receptors and calcium-dependent growth signals (14–17). When activated, CADTK appears to stimulate tyrosine phosphorylation of cytoskeleton proteins and activate intracellular signaling. For example, our data and the data of others suggest that CADTK tyrosine-phosphorylates paxillin, tensin, and p130CAS and forms complexes with paxillin and p130(CAS) (35, 36, 57). In GN4 cells, CADTK also appears to stimulate two protein kinase cascades leading to JNK and p70S6K activation (14, 27) and Tokiwa et al. (18) have directly demonstrated a CADTK/PYK2 to JNK activation pathway. In PC12 cells calcium-dependent CADTK/PYK2 activation regulates ERKs (15, 37), but in rat liver epithelial cells, the pure calcium signal produced by thapsigargin does not result in Raf, ERK, or p90RSK activation (14). Interestingly, in T lymphocytes, a calcium signal alone is not sufficient to activate JNK (38), although CADTK is expressed in the Jurkat cell line (17). We are currently working to uncover the bases for these cell type specificities, but in summary, CADTK is a potential mediator of G-protein-coupled receptor and calcium signal-regulated alterations in cell shape and gene expression.

The biological relevance of this PKC attenuation was uncovered by examining agonist-dependent c-Jun and c-Fos expression (Fig. 5). c-Jun has a relatively simple promoter, and most inducers activate its expression through one major cis-element. The phosphor-ylated inducing inducers activate its expression through one major cis-element, and ATF2 are phosphorylated, stimulating their ability to transactivate and increase c-Jun expression (42). The phosphor-ylated inducing inducers activate its expression through one major cis-element, and gene expression.

The present study begins to define the calcium/CADTK pathway to JNK and to clearly differentiate this JNK activation pathway from the better studied stress-dependent pathway initiated by anisomycin, UV, etc. Our results show that calcium-dependent JNK activation is inhibited by TPA-sensitive PKC, while the stress pathway is not (Fig. 3). Since TPA does not inhibit thapsigargin-dependent CADTK tyrosine phosphorylation (Fig. 4), the site of PKC action is distal to CADTK. The inhibition by PKC, even when initiated 1–2 min after a calcium signal (Figs. 2 and 3), demonstrates why thapsigargin, which does not stimulate CADTK activity as well as Ang II (Fig. 4), activates JNK more potently (Fig. 1). The better CADTK stimulus, Ang II, produces both positive (CADTK) and negative (PKC) signals to the JNK pathway, while thapsigargin produces only a positive signal.

The biological relevance of this PKC attenuation was uncovered by examining agonist-dependent c-Jun and c-Fos expression (Fig. 5). c-Jun has a relatively simple promoter, and most inducers activate its expression through one major cis-element, the c-Jun AP-1 site (39). This AP-1 site differs from the consensus AP-1 sequence due to a 1-base pair insertion, resulting in preferential recognition by c-Jun-ATF2 heterodimers rather than the conventional AP-1 complex (Jun-Fos) (40). Furthermore, ATF2 is preferentially activated by JNK to mediate c-Jun induction in response to some stimuliators (41). c-Jun AP-1 is constitutively occupied in vivo, and the occupying c-Jun and ATF2 are phosphorylated, stimulating their ability to transactivate and increase c-Jun expression (42). The phosphorylation-induced c-Jun expression provides newly synthesized c-Jun, continuing the induction. In GN4 cells, Ang II-induced c-Jun expression lasted for about 60 min and was weaker than that of thapsigargin. In contrast, the expression induced by thapsigargin increased steadily for 2 h (Fig. 5). These data are
PKC and PKA Inhibit Calcium-dependent JNK Activation

most readily explained by the negative regulation of JNK activation by PKC in Ang II-treated cells. We have not formally ruled out cessation of Ang II c-Jun induction by ERK phosphorylation of an inhibitory site within the c-Jun carboxyl-terminal DNA binding domain (42). However, induction of c-Jun by EGF, a maximal activator of ERK, was greater and more sustained than that of Ang II but not as prolonged as that of thapsigargin. This suggests that ERKs do not inhibit c-Jun expression in GN4 cells and that the inhibitory effect of PKC on the CADTK to JNK pathway is not due to its secondary activation of ERKs (Fig. 3C). The EGF-dependent pathway probably involves a mechanism in addition to JNK, since EGF is a poor activator of JNK in these cells (Fig. 3C) (11).

The 40–50-fold thapsigargin-dependent JNK activation begins to decrease after 30 min, while c-jun expression continues upward for at least 2 h. The explanation may lie in the continued, unopposed 20-fold JNK activation and the two phases of c-Jun induction (JNK phosphorylation of pre-existing c-Jun and ATF2 and the effect of newly synthesized transcriptional factors). It has been reported that JNK can also mediate Elk-1 phosphorylation inducing c-Fos expression (10). However, this phenomenon must be minimal in GN4 cells, because substantial thapsigargin-dependent JNK activation leads only to barely detectable c-Fos expression (Fig. 5).

The physiological consequence of constitutively increasing c-Jun expression may vary in different cell types. In differentiated PC12 cells, the withdrawal of NGF results in apoptosis. NGF withdrawal correlated with the activation of the JNK and p38 MAPK and decreased ERK activity. Thus, in these cells persistent activation of the JNK and/or p38 MAPK pathways promotes apoptosis particularly in these stress activated enzymes and are not accompanied by an increase in ERK (43). Persistent JNK activation has also been implicated in ceramide-, Fas-, and γ radiation-induced apoptosis (44–47). However, activation of JNK mediated by CD40 ligation in B cells protects against, rather than stimulates, apoptosis (48, 49).

In addition, JNK activation is not involved in induction of apoptosis by tumor necrosis factor-α (50), and competitive inhibitory mutant JNK does not attenuate MEKK1-stimulated cell apoptosis in Swiss 3T3 cells (51). In GN4 cells, Ang II activates both ERKs and JNK balancing c-Fos and c-Jun expression and leading to cell proliferation (31). Thapsigargin, which strongly activates JNK but not ERK, disrupts the balance between c-Fos and c-Jun expression, but it remains to be determined whether thapsigargin promotes apoptosis and whether TPA could block this putative thapsigargin action. There are examples of di-glyceride attenuation of ceramide-induced apoptosis in human myeloid leukemia cells (52, 53) and sphingosine-1-phosphate suppression of ceramide-mediated cell death (45) which could involve PKC-dependent attenuation of a CADTK to JNK pathway.

In GN4 cells, increases in cAMP also blocks calcium-dependent JNK activation. These findings are consistent with recent reports that cAMP inhibits thrombin-induced JNK activity in vascular smooth muscle cells (25, 26) and in T lymphocytes (24), since both T cells (17) and rat aortic smooth muscle cells (28) express CADTK/RAFTK. CADTK is activated by platelet-derived growth factor and Gα coupled receptors, e.g. Ang II (28) and this activation is correlated with JNK activation in rat aortic smooth muscle cells. (28) The target for cAMP-dependent inhibition may be downstream from the component inhibited by PKC, or it may simply take longer to develop.

Based on our data, the model in Fig. 9 is proposed. Thapsigargin and Ang II produce a calcium signal and by an unknown mechanism activate CADTK. This presumably sends a positive signal to both the JNK (11, 14) and p70S6K (27) pathways. Ang II also stimulates PKC, sending an inhibitory signal to the JNK pathway. The calcium-dependent pathway to p70S6K passes through wortmannin and rapamycin-sensitive steps, which are well characterized in many cell types, but neither wortmannin nor rapamycin inhibited CADTK tyrosine phosphorylation or the calcium-dependent JNK pathway. It is interesting to note that TPA, which does stimulate JNK (54) in some cell types, does not activate these enzymes in GN4 cells; rather, PKC (or specific isoforms) are utilized in an inhibitory network regulating the extent and duration of JNK activation. Last, while evidence of a calcium/CADTK to JNK pathway is substantial, we must be somewhat circumspect about the CADTK to p70S6K pathway until more direct evidence is obtained.

A more complete understanding of the effect of PKC on inhibition of the CADTK to JNK pathway (as opposed to the stress-dependent pathway) will only come by defining pathway components. Stress-dependent JNK activation appears to be mediated by small GTPase proteins, such as Rac and CDC42H and PAK-like protein kinases that may turn on activate the MEKK/stress-activated protein kinase/JNK pathway (2, 7). Recently, PAK1 and -2 were shown to be activated by chemoattractants (55). These PAKs may mediate a JNK activation signal by heterotrimeric G-protein-coupled receptors and their G-protein components, e.g. βγ subunits (56). Since thapsigargin mimics the effect of G-protein-coupled receptors in GN4 cells, it is unlikely that βγ are involved. Whether the CADTK to JNK pathway uses PAK-like enzyme is unknown, but our best clue to the mechanism at this point is that the calcium/CADTK pathway uses some elements distinct from the stress-dependent pathway.

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