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An Intracellular Calcium Signal Activates p70 but Not p90 Ribosomal S6 Kinase in Liver Epithelial Cells*

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In the rat liver epithelial cell lines GN4 and WB, angiotensin II (Ang II) activates the G_q class of regulatory G-proteins, increasing intracellular calcium, protein kinase C activity, and protein tyrosine phosphorylation. We compared the ability of Ang II and other compounds that increase intracellular calcium (*i.e.* the calcium ionophore A23187 and thapsigargin) or protein kinase C activity (the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate) to activate p70 ribosomal S6 kinase ($p70^{S6K}$) and p90 ribosomal S6 kinase ($p90^{RSK}$). In GN4 cells, increasing intracellular calcium stimulated $p70^{S6K}$ activity in a rapamycin- and wortmannin- sensitive manner, but did not affect $p90^{RSK}$ activity. In contrast, 12-*O*-tetradecanoylphorbol-13-acetate strongly activated $p90^{RSK}$ but only weakly stimulated $p70^{S6K}$. The ability of calcium to activate $p70^{S6K}$ was confirmed by blocking the A23187-dependent activation through chelation of extracellular calcium with EGTA; the effect of thapsigargin was inhibited by the cell permeant chelator bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM). Similarly, BAPTA-AM prevented the activation of $p70^{S6K}$ by Ang II, suggesting that this signal was largely calcium-dependent. In contrast, the Ang II-dependent activation of mitogen-activated protein kinase and $p90^{RSK}$ was not inhibited but was enhanced by BAPTA-AM. These results show that in GN4 cells, Ang II selectively activates $p70^{S6K}$ through effects on calcium, $p90^{RSK}$ through effects on protein kinase C. The activation of $p70^{S6K}$ by calcium stimuli or Ang II was independent of calmodulin but correlated well with the activation of the recently identified, nonreceptor calcium-dependent tyrosine kinase (CADTK)/PYK-2. Both calcium- and Ang II-dependent activation of $p70^{S6K}$ were attenuated by the tyrosine kinase inhibitor genistein, and activation of $p70^{S6K}$ was higher in GN4 than WB cells, correlating with the increased expression and activation of CADTK/PYK-2 in GN4 cells. In summary, these results demonstrate that intracellular calcium selectively activates $p70^{S6K}$ in GN4 cells, consistent with increased CADTK/PYK-2 signaling in these cells.

The peptide hormone angiotensin II (Ang II)[1] initiates multiple intracellular processes that in some cell types influence cell proliferation. The seven-transmembrane-spanning Ang II receptor is coupled to the G_q class of regulatory G-proteins, which, when activated, stimulates inositol phosphate-mediated calcium release, protein kinase C activity, and additional signals regulated by $\beta\gamma$ subunits (1). In rat liver epithelial cells, an additional response to Ang II is a rapid (15–60 s), calcium-dependent increase in tyrosine phosphorylation of proteins (2, 3). One of the tyrosine-phosphorylated proteins has been purified from Ang II-treated rat liver epithelial cells using ATP affinity chromatography as the penultimate step and shown to be the major calcium-dependent protein tyrosine kinase (CADTK) in these cells (4). Although not directly activated by calcium *in vitro*, this kinase is stimulated by treatment of cells with agonists and hormones that elevate intracellular calcium (3, 4). Peptide sequencing of the purified kinase and cDNA cloning using rat liver epithelial cell mRNA revealed a novel cytoplasmic tyrosine kinase highly related to the p125 focal adhesion kinase (5). The kinase has also been recently identified by three other groups. One group using a polymerase chain reaction strategy to search for novel kinases cloned the human sequence PYK-2 and have also shown its activation in a calcium- and PKC-dependent manner (6). Two other groups screening for kinases related to p125 focal adhesion kinase have reported the sequence as cell adhesion kinase β and related adhesion focal tyrosine kinase (7, 8).

In addition to stimulation of calcium and calmodulin, PKC, MAPK, c-Jun N-terminal protein kinase (JNK), and CADTK/PYK-2 (1, 9, 10), Ang II has been shown to activate the ribosomal p70 S6 kinase ($p70^{S6K}$) (11). This ubiquitous class of mitogen-activated protein kinases is best known for their ability to phosphorylate the S6 protein of the 40 S ribosome (reviewed in Ref. 12). Although related in sequence to another S6 kinase ($p90^{RSK}$), the regulation of $p70^{S6K}$ is clearly distinct from the $p90^{RSK}$, the latter being activated as part of the Ras-dependent MAPK cascade (13). In contrast to $p90^{RSK}$, activation of the $p70^{S6K}$ pathway is inhibited by rapamycin (14, 15). In mammals, the intracellular target for rapamycin is the binding protein FKB12, which forms a complex with the recently identified protein kinases known as FRAP and RAFT (16, 17). Similar in sequence homology to the phosphatidylinositol-3

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[1] The abbreviations used are: Ang II, angiotensin II; MAPK, mitogen-activated protein kinase; BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester; CADTK, calcium-dependent tyrosine kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PI-3 kinase, phosphatidylinositol-3 kinase; EGF, epidermal growth factor; TBST, Tris-buffered saline/Tween; PBS, phosphate-buffered saline; PKC, protein kinase C; $p70^{S6K}$, p70 ribosomal S6 kinase; $p90^{RSK}$, p90 ribosomal S6 kinase; JNK, c-Jun kinase; PAGE, polyacrylamide gel electrophoresis; RIPA, radioimmunoprecipitation assay; RAFT, rapamycin and FKBP12 target; FRAP, FKBP-rapamycin-associated protein.

kinases (PI-3 kinases) (18), the FRAP/RAFT kinase is required for activation of $p70^{S6K}$ (16) but does not appear to phosphorylate phosphatidylinositol, PI-3 kinase, or $p70^{S6K}$. Thus the mechanism by which FRAP/RAFT regulates $p70^{S6K}$ signaling remains to be elucidated.

The $p70^{S6K}$ can be activated by numerous stimuli, including growth factors, inhibitors of protein synthesis (cycloheximide or anisomycin), and, as noted above, hormones such as Ang II (11, 19, 20). Complete activation of $p70^{S6K}$ appears to require multisite phosphorylation by several protein kinases, at least one of which is proline-directed and phosphorylates a sequence(s) in the C-terminal region of the S6 kinase (21–24). Despite the knowledge of specific phosphorylation sites, the activating "S6 kinase kinases" remain to be identified. In addition to the involvement of the rapamycin-sensitive FRAP/RAFT mentioned above, $p70^{S6K}$ is inhibited by wortmannin and LY294002, implicating PI-3 kinase in the signaling to $p70^{S6K}$ (25, 26). These results are further supported by studies involving expression of a constitutively active PI-3 kinase (27). A potential intermediary for PI-3 kinase in $p70^{S6K}$ activation is the serine and threonine kinase PKB, also known as Akt (28, 29), which has been shown to activate $p70^{S6K}$ when co-expressed in cells (30).

The $p70^{S6K}$ is involved in mitogenesis; studies with neutralizing antibodies to $p70^{S6K}$ or the inhibitor rapamycin have shown that this kinase is required for G_1 progression (31–33). The ability of $p70^{S6K}$ to phosphorylate the ribosomal S6 protein has been well characterized (34), but the contribution of this event to the regulation of protein synthesis is less clear (reviewed in Ref. 12). Phosphorylation of the S6 protein has been found to enhance the translation of mRNAs containing 5′-polypyrimidine tracts (35, 36), and it is likely that $p70^{S6K}$ plays a role in growth-related control of translation. For example, translation of specific messages such as those encoding elongation factor 1α (36) or insulin-like growth factor II (37) appear to be regulated by the S6 pathway; these may be members of a class of proteins required for progression through the G_1 phase of the cell cycle.

Ang II also stimulates protein synthesis in a number of cell types (11, 38), an event that may be dependent on calcium (39) and $p70^{S6K}$ (11). Because of our interest in calcium and Ang II signaling, we compared the ability of these stimuli to activate the $p70^{S6K}$ in liver epithelial cells, cells known to have a proliferative response to Ang II (1). In addition, Ang II leads to a protein kinase C-independent increase in AP1 binding, as well as stimulation of MAP kinase and c-Jun N-terminal kinase (10) and a calcium-dependent activation of a novel; calcium-dependent tyrosine kinase (CADTK). In this article we describe the finding that agents and hormones that increase intracellular calcium activate $p70^{S6K}$ but not $p90^{RSK}$. Furthermore, these studies suggest that the initial steps in Ang II action are well correlated with activation of CADTK.

EXPERIMENTAL PROCEDURES

Materials—Human Ang II (DRVYIHPF) was obtained from Sigma and prepared in 50 mM acetic acid prior to use. The S6 peptide (RRLSSLRA) (40) and PKI peptide (TTYADFIASGRTGRRNAIHD) (41) were synthesized by Dr. D. Klapper (University of North Carolina, Chapel Hill, NC). Thapsigargin, A23187, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calyculin A, wortmannin, rapamycin, anisomycin, and calmidizolium were obtained from Biomol and prepared in Me_2SO. Calmodulin, leupeptin, and aprotinin were obtained from Sigma. Epidermal growth factor (EGF) was obtained and used as described previously (10). The intact 40 S ribosomal subunit was purified from rat liver by the procedure of Terao and Ogata (42).

Cell Culturing and Harvesting—Rat liver epithelial cells (GN4 or WB) were grown at 37 °C in Richter's minimal essential media containing 0.1 μM insulin and supplemented with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere as described earlier (3). Cells were serum starved (0.1% fetal bovine serum) for 20–24 h prior to agonist stimulation. Cells were washed twice with phosphate-buffered saline (PBS) and once with Buffer H (50 mM β-glycerophosphate, pH 7.4, 1.5 mM EGTA, 0.15 mM sodium orthovanadate, 1 mM dithiothreitol, 25 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), after which they were scraped in 0.5 ml of Buffer H. The cells were then sonicated with two 5-s pulses (Fisher MDL 550 Sonic Dismembrator), and the lysates were centrifuged at 100,000 $\times$ *g* (4 °C).

$p70^{S6K}$ Assays—The activity of $p70^{S6K}$ in cell lysates was determined as described earlier (43). The 100,000 $\times$ *g* supernatants were assayed for $p70^{S6K}$ activity by monitoring the phosphorylation of the ribosomal protein S6 (2 μg) in a buffer (30 μl) containing 25 mM β-glycerophosphate (pH 7.4), 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 μM calmidizolium, 2 μM PKI peptide, and 100 μM [γ-^{32}P]ATP (2000 μCi/mmol) for 20 min at 30 °C. The reactions were terminated by the addition of SDS-PAGE sample buffer (30 μl), heated, and applied to SDS-PAGE (10% acrylamide). The radioactive band corresponding to the ribosomal S6 protein was identified by staining with Coomassie Blue and autoradiography; the radioactive band was excised from the dried gel and quantitated by liquid scintillation counting. Calmidizolium (10 μM) was included in the assay mixture to prevent the phosphorylation of the ribosomal S6 protein by calcium- and calmodulin-dependent kinases.

Immunoprecipitation Assays for $p70^{S6K}$ and $p90^{RSK}$—In some experiments, $p70^{S6K}$ was assayed by immunoprecipitation of the $p70^{S6K}$ after lysis of the cells in Tris/Triton lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 nM calyculin A, and 148 μM Na_3VO_4). The samples were immunoprecipitated as described below and the immunoprecipitate/protein A-agarose beads were washed once with Tris/Triton lysis buffer and twice with PBS (1 ml). The S6 kinase assays were performed using a modification of the above assay procedure. In these assays, the immunoprecipitate/protein A-agarose beads (20 μL) were assayed in 20 mM Hepes, pH 7.3, 10 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 mM $MgCl_2$, and 50 μM [γ-^{32}P]ATP (1000 μCi/mmol) plus 250 μM S6 peptide in a final reaction volume of 60 μl. The assay was performed at 30 °C for 20 min with constant shaking in an Eppendorf 5436 Thermomixer. The reaction was terminated by the addition of 20 μl of 100 mM EDTA (pH 7.0); the samples were centrifuged for 5 min at 13,000 $\times$ *g*, and the supernatant (40 μl) was spotted on Whatman P-81 paper. The papers were washed in 10% phosphoric acid, and the radioactivity incorporated into S6 peptide was determined by liquid scintillation counting. The assays for $p90^{RSK}$ were performed exactly as described above, except the cell lysates were immunoprecipitated with a rabbit polyclonal antibody developed against the C terminus of $p90^{RSK}$ as described previously (Ref. 44; kindly provided by Dr. Edwin G. Krebs, Department of Pharmacology, University of Washington).

$p70^{S6K}$ Immunoprecipitation and Immunoblotting—For immunodetection of $p70^{S6K}$, cells were washed as described above and lysed in RIPA buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1.0% Triton X-100, and 2.0 mM EDTA plus 10 nM calyculin A and 0.5 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,000 $\times$ *g* for 10 min (4 °C). The samples were immunoprecipitated using a rabbit polyclonal antibody developed against a C-terminal peptide (QAFPMISKRPEHLRMNL) of the $p70^{S6K}$ (kindly provided by Dr. J. Weiel, Glaxo-Wellcome). Immunoprecipitation was facilitated by the addition of protein A-agarose (20 μl, packed beads), and the immunoprecipitates were washed three times with RIPA buffer prior to addition of SDS-PAGE sample buffer. Samples were applied to SDS-PAGE and blotted to polyvinylidene difluoride, and the immunoprecipitated $p70^{S6K}$ was detected by incubating blots with the C-terminal $p70^{S6K}$ antibody (diluted 1:2500 in TBST). The immunoblot was incubated with goat anti-rabbit (alkaline phosphatase-conjugated) antibodies (diluted 1:5000 in TBST), and the color was developed according to the manufacturer's procedure (Promega).

Measurement of CADTK Tyrosine Autophosphorylation—The activity of CADTK was assayed by determining the amount of tyrosine autophosphorylation by a modification of the procedure described earlier (4). Briefly, the cells were washed twice with PBS and harvested in RIPA or Tris/Triton lysis buffer as described above for the S6 kinase immunoblotting experiments. The CADTK was immunoprecipitated with a rabbit polyclonal antibody developed against a glutathione *S*-transferase-CADTK fusion protein (5). The CADTK immunoprecipitates were washed three times with RIPA or Tris/Triton lysis buffer and applied to SDS-PAGE (8% acrylamide). After electrophoresis the pro-

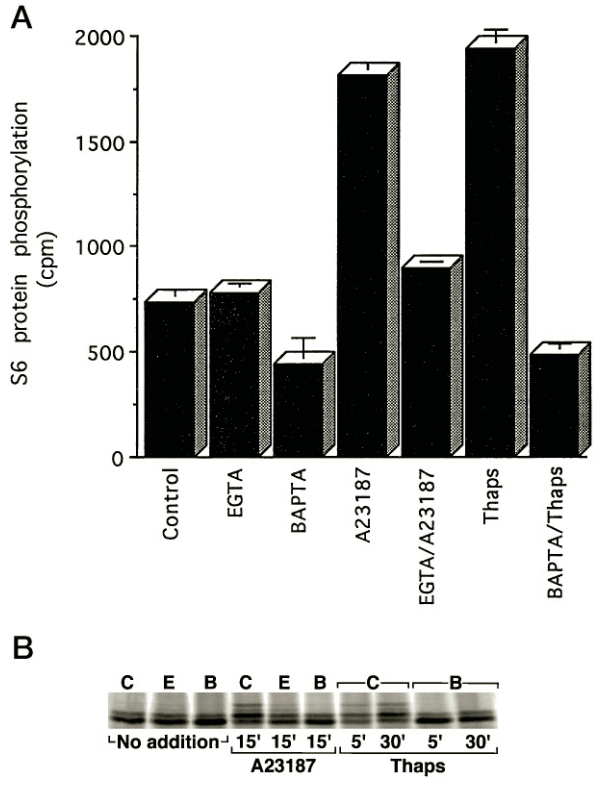

FIG. 1. **Calcium agonists activate p70^{S6K}.** *A,* GN4 cells were grown in 10-cm dishes and serum-starved as described under "Experimental Procedures." The cells were then incubated with 1 μM A23187 or 2 μM thapsigargin (*Thaps*) for 10 min. In some experiments, the calcium chelators EGTA (5 mM) and BAPTA (50 μM) were added to the medium 15 min prior to the addition of calcium stimuli. The cells were harvested and assayed for S6 kinase activity as described under "Experimental Procedures," and the S6 kinase activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein. The results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 2$ experiments. *B,* cells were prepared as described in *A* and treated with calcium agonists for the lengths of time indicated at the *bottom*. In some experiments cells were incubated with PBS (*C*), 5 mM EGTA (*E*), or 50 μM BAPTA (*B*) for 15 min prior to the addition of calcium stimuli. The cell lysates were prepared by washing the cells twice with PBS and adding 1 ml of RIPA buffer to solubilize the cells. The cell lysate was clarified by centrifugation at 12,000 × *g* prior to the addition of antisera to p70^{S6K}. The immunoprecipitated p70^{S6K} was identified by SDS-PAGE and immunoblotting as described under "Experimental Procedures."

teins were transferred electrophoretically to nitrocellulose, and the amount of phosphotyrosine on CADTK was quantitated by antiphosphotyrosine immunoblotting (PT66) and ECL as described earlier (4).

RESULTS

Calcium Activates p70^{S6K} but Not p90^{RSK}—Incubation of GN4 rat liver epithelial cells with compounds that increase intracellular calcium (*i.e.* the ionophore A23187, thapsigargin (which releases calcium from internal stores; Ref. 45), or Ang II), stimulates intracellular protein tyrosine phosphorylation (2). To investigate the influence of calcium-stimulated tyrosine phosphorylation on "downstream" kinase signaling pathways, we examined the ability of calcium to activate the ribosomal S6 kinase p70^{S6K}. Incubation of GN4 cells with 1 μM A23187 or 2 μM thapsigargin increased S6 kinase activity as measured by the phosphorylation of the 40 S ribosomal S6 protein (Fig. 1*A*). A typical response to these stimuli was a 2–3-fold increase over basal levels, similar to that produced by EGF (See Fig. 3). The calcium-stimulated increase in S6 kinase activity was inhibited by the calcium chelators EGTA and BAPTA-AM. Preincubation with EGTA prevented activation of p70^{S6K} by A23187; the cell-permeant chelator BAPTA-AM (BAPTA) inhibited both the thapsigargin- and ionophore-stimulated S6 kinase activity (Fig. 1*A*). These results indicate that for these agonists the increase in p70^{S6K} activity is calcium-dependent, and with thapsigargin, the effect is primarily dependent on intracellular calcium.

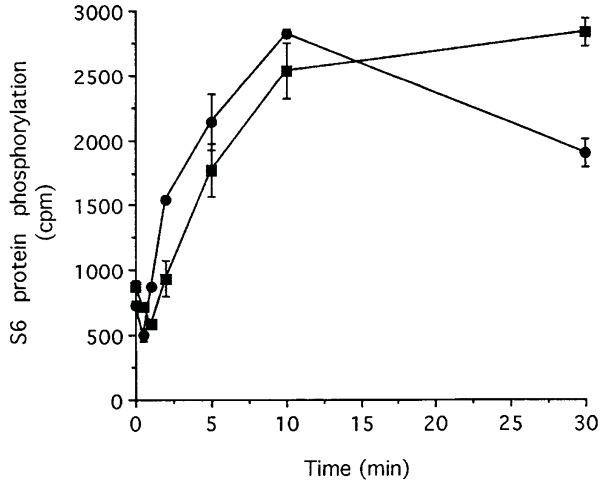

FIG. 2. **Angiotensin II and thapsigargin increase p70^{S6K} activity.** GN4 cells were prepared as described in the legend to Fig. 1 and incubated with 1 μM Ang II (●) or 2 μM thapsigargin (■) for the lengths of time indicated. The cells were harvested, and the cell lysates were assayed for S6 kinase activity as described earlier. The S6 kinase activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein, and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments.

In addition to the increased the phosphorylation of the ribosomal S6 protein, calcium activated p70^{S6K} as demonstrated by SDS-PAGE and immunoblotting analysis (Fig. 1*B*). In these experiments, p70^{S6K} was immunoprecipitated from cell lysates and immunoblotted for p70^{S6K} as described under "Experimental Procedures." Four immunoreactive bands corresponding to the multiple phosphorylated forms of p70^{S6K} were detected (46). In all experiments, increased p70^{S6K} activity (as determined by S6 protein phosphorylation) correlated with an upward mobility shift of both the slowest and fastest migrating immunoreactive bands on SDS-PAGE. The increased upward mobility shift of p70^{S6K} produced by A23187, or thapsigargin was prevented by preincubation with calcium chelators (Fig. 1*B*). BAPTA preincubation reduced the mobility shift of p70^{S6K} from untreated and A23187- or thapsigargin-treated cells, demonstrating that BAPTA affected both the stimulated and basal S6 kinase activity. In contrast, EGTA inhibited only the A23187-stimulated S6 kinase mobility shift, similar to the results obtained by assaying S6 protein phosphorylation. (Fig. 1, *A* and *B*).

Like thapsigargin, Ang II rapidly increases intracellular calcium and tyrosine phosphorylation in rat liver epithelial cells (2). As shown in Fig. 2, incubation of GN4 cells with Ang II rapidly stimulated p70^{S6K} activity comparable with that observed with thapsigargin. The increase in p70^{S6K} activity was apparent as early as 2–5 min after Ang II addition, with the peak of S6 phosphorylation occurring after 10–20 min. The effect of Ang II and thapsigargin was sustained for more than 60 min (data not shown). Similar results were found by analyzing immunoblots of p70^{S6K}; Ang II, thapsigargin, and A23187 stimulated a time-dependent increase in the p70^{S6K} mobility shift on SDS-PAGE, which paralleled the increase in S6 protein phosphorylation (see Fig. 4*A*).

In cardiac myocytes, Ang II and calcium have been reported to stimulate the activity of MAPK and the ribosomal S6 kinase p90^{RSK} (47). Although Ang II activated MAPK in GN4 cells by

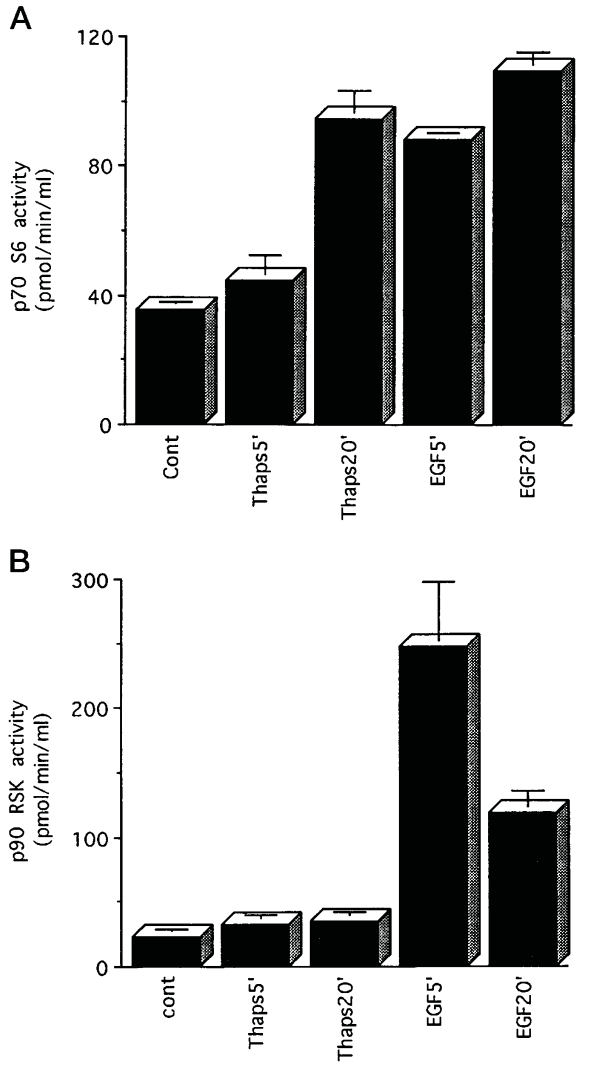

FIG. 3. **Thapsigargin activates p70^{S6K}, but not p90^{RSK}.** Serum-starved GN4 cells were incubated with 2 μM thapsigargin (*Thaps*) or 6.6 nM EGF for the lengths of time indicated. The cells were washed two times with PBS and harvested in Tris/Triton lysis buffer as described under "Experimental Procedures." The lysate from each 10-cm dish was immunoprecipitated with antibodies specific for p70^{S6K} (*A*) or p90^{RSK} (*B*) and assayed for S6 peptide kinase activity as described above. The S6 peptide activity is plotted as the amount of activity (pmol/min/ml), and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *cont,* control.

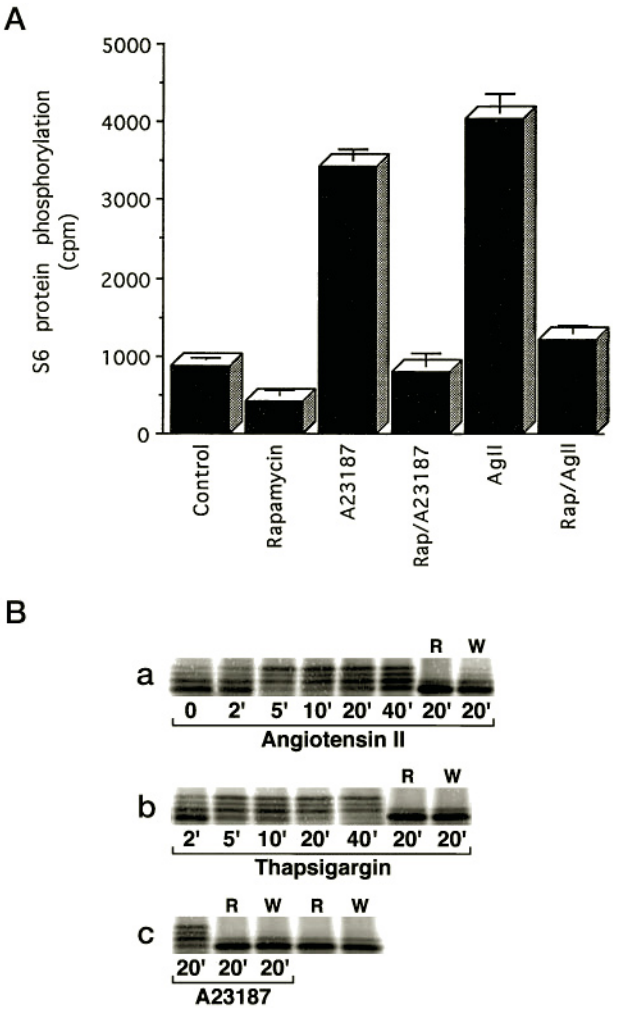

FIG. 4. **Rapamycin and wortmannin inhibit the activation of p70^{S6K} by calcium agonists.** *A,* GN4 cells were incubated with 0.1% Me_2SO (carrier for rapamycin) or rapamycin (*Rap,* 10 nM, 15 min) prior to the addition of A23187 (1 μM) or Ang II (*AgII,* 1 μM). Cell lysates were prepared as described earlier, and the S6 kinase activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein. The results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *B,* GN4 cells were incubated with 1 μM Ang II, 2 μM thapsigargin or 1 μM A23187 for the lengths of time indicated. In some experiments, cells were incubated with 10 nM rapamycin (*R*) or 50 nM wortmannin (*W*) for 15 min prior to the addition of agonist. The p70^{S6K} was immunoprecipitated and detected by immunoblotting as described in the legend to Fig. 1.

a PKC-dependent mechanism, thapsigargin does not stimulate MAPK in these cells (5, 10). We therefore compared the ability of thapsigargin and EGF to activate p70^{S6K} and p90^{RSK} in GN4 cells. As expected, both agonists stimulated p70^{S6K} (Fig. 3*A*), but only EGF stimulated p90^{RSK} in GN4 cells (Fig. 3*B*). The kinase assay on p90^{RSK} immunocomplexes using the S6 peptide (RRLSSLRA) as a substrate also demonstrated that A23187 did not activate p90^{RSK}, whereas Ang II potently activated this enzyme, as previously reported (10) (data not shown).

Calcium-dependent p70^{S6K} Activation Is Inhibited by Wortmannin and Rapamycin—To further rule out the possibility that p90^{RSK} was contributing to the phosphorylation of the S6 protein in cell lysates and to investigate the mechanism by which calcium stimulates p70^{S6K}, we examined the effect of compounds known to inhibit p70^{S6K} activation. Rapamycin has been previously shown to inhibit p70^{S6K} without affecting p90^{RSK} activity (14, 15). As shown in (Fig. 4*A*), incubation of cells with 10 nM rapamycin completely inhibited the Ang II- and calcium ionophore (A23187)-stimulated increase in p70^{S6K} S6 peptide kinase activity. Similarly, rapamycin blocked the thapsigargin-dependent activation of p70^{S6K}; (unstimulated, 35.7 ± 0.4 pmol/min/ml; thapsigargin, 94.3 ± 7.1 pmol/min/ml; thapsigargin and rapamycin, 23.9 ± 0.2 pmol/min/ml). Rapamycin also blocked Ang II-, A23187-, and thapsigargin-dependent p70^{S6K} activation, as assessed by the gel mobility immunoblotting (Fig. 4*B*). These results demonstrate that Ang II and calcium stimulate p70^{S6K} through a rapamycin-sensitive pathway and confirm that p70^{S6K} and not p90^{RSK} is the major ribosomal S6 kinase activated by calcium stimuli in GN4 cells.

Studies with the inhibitor wortmannin and constitutively active forms of PI-3 kinase have demonstrated a role for this enzyme in the regulation of p70^{S6K} (25–27). To determine whether calcium stimulated p70^{S6K} through a PI-3-kinase-dependent pathway, the effect of wortmannin on p70^{S6K} activity was examined. As shown in Fig. 4*B*, incubation of GN4 cells with wortmannin (50 nM) inhibited the mobility shift of p70^{S6K} stimulated by Ang II, A23187, or thapsigargin. The inhibition

of $p70^{S6K}$ by wortmannin occurred at low concentrations (10–50 nM), consistent with the effects of this compound on PI-3 kinase (48). Similar inhibitory affects of wortmannin were found on the calcium-dependent activation of S6 kinase, as determined by S6 protein phosphorylation (data not shown). Thus, this calcium-stimulated signaling pathway appears to be mediated through both a PI-3 kinase and FRAP/RAFT-dependent pathway.

Angiotensin II Activates $p70^{S6K}$ in a Calcium-dependent Manner—Ang II stimulates an inositol phosphate-mediated increase in intracellular calcium (reviewed in Ref. 49), and since our results demonstrated that calcium stimulates $p70^{S6K}$ activity, we examined whether the activation by Ang II was calcium-dependent. Incubation of GN4 cells with the calcium chelator BAPTA inhibited the Ang II-stimulated S6 kinase activity in a dose-dependent manner, with >90% inhibition occurring at 50 μM BAPTA (Fig. 5*A*). Like the results found with S6 kinase activity, BAPTA inhibited the Ang II-stimulated $p70^{S6K}$ mobility shift (Fig. 5*B*) at concentrations similar to those required to inhibit the activation by A23187 or thapsigargin (Fig. 1, *A* and *B*). Chelation of extracellular calcium with EGTA (5 mM) only minimally affected the Ang II-stimulated S6 kinase activity, further supporting the thesis that intracellular calcium was required for the activation of $p70^{S6K}$ (Fig. 5*B*). The calcium-dependent activation of $p70^{S6K}$ occurred independently of calmodulin, since the calmodulin inhibitor calmidizolium (50) did not inhibit this event. Similarly, neither calmidizolium nor W-7 prevented the autophosphorylation of CADTK, although calmidizolium (15 μM) inhibited the Ang II-dependent activation of the myosin light chain peptide kinase activity, a known calmodulin-dependent process (data not shown). In comparison, preincubation of GN4 cells with BAPTA did not inhibit but significantly stimulated the activation of $p90^{RSK}$ by Ang II in a dose-dependent manner (Fig. 5*C*). Furthermore, incubation with BAPTA alone (50 μM) increased the basal level of $p90^{RSK}$ to levels equivalent to that of Ang II stimulation in these cells. Similar to the results obtained with $p90^{RSK}$, BAPTA pretreatment potently enhanced the basal and Ang II-stimulated level of MAPK activity (data not shown).

PKC Activates $p90^{RSK}$ but Not $p70^{S6K}$ in GN4 Cells—Since Ang II increases diacylglycerol formation and PKC activity in GN4 cells, we investigated whether the activation of $p70^{S6K}$ by Ang II was also PKC-dependent. Incubation of cells with phorbol ester (TPA, 100 nM) for 20 min did not stimulate $p70^{S6K}$ activity in these cells, although $p90^{RSK}$ was stimulated by TPA (100 nM, 5 min) (Fig. 6, *A* and *B*). To further investigate the involvement of PKC, PKC activity was down-regulated by chronic incubation with TPA (1 μM, 24 h). The activation of $p70^{S6K}$ by Ang II was not inhibited by this treatment, indicating that PKC did not play a substantial role in regulating $p70^{S6K}$ in these cells (Fig. 6*A*). In comparison, this treatment effectively eliminated the activation of $p90^{RSK}$ by TPA as expected (Fig. 6*B*).

The Activation of $p70^{S6K}$ by Calcium Requires a Tyrosine Kinase—In epithelial cells, the Ang II- and calcium-stimulated increase in protein tyrosine phosphorylation is inhibited by the tyrosine kinase inhibitor genistein (3, 51). GN4 cells were briefly incubated with genistein (10–200 μM) to test the ability of this compound to prevent the activation of $p70^{S6K}$ by Ang II or calcium stimuli. As shown in Fig. 7*A*, genistein inhibited the Ang II-stimulated $p70^{S6K}$ activity in a dose-dependent manner, suggesting the involvement of a tyrosine kinase in the calcium and Ang II signaling to $p70^{S6K}$. Similarly, genistein inhibited the activation of $p70^{S6K}$ by thapsigargin (data not shown). Recently, we have purified the major calcium- and Ang II-stimulated tyrosine kinase (CADTK) from GN4 rat liver epi-

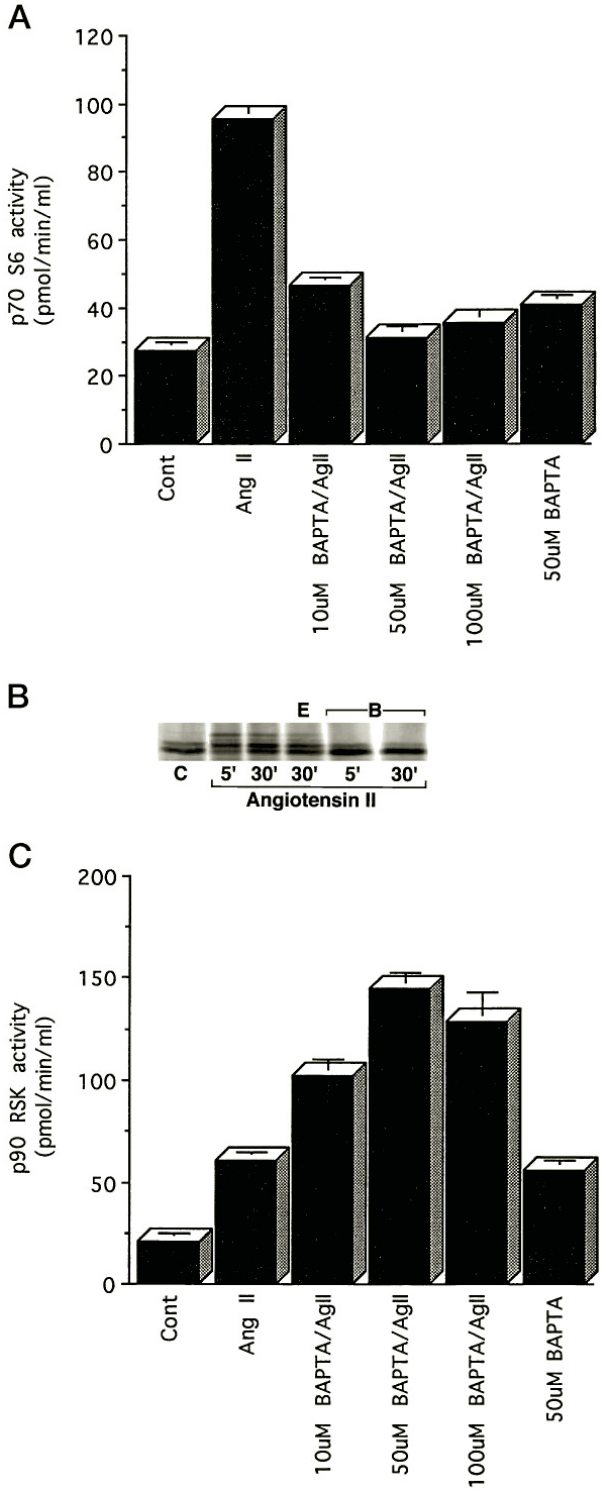

FIG. 5. **Angiotensin II activates $p70^{S6K}$ in a calcium-dependent manner.** *A,* $p70^{S6K}$ activity. Serum-starved GN4 cells were incubated with 1 μM Ang II for 20 min; in some experiments cells were incubated with the indicated concentrations of BAPTA for 15 min prior to the addition of Ang II (*AgII*). Cell lysates were prepared, and the $p70^{S6K}$ was immunoprecipitated and assayed as described earlier. The $p70^{S6K}$ activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (*bars*) of duplicate samples. *B,* serum-starved GN4 cells were incubated with Me_2SO (carrier), 5 mM EGTA (*E*), or 50 μM BAPTA (*B*) for 15 min prior to the addition of Ang II (1 μM) for the amount of time indicated; $p70^{S6K}$ was analyzed for activity by SDS-PAGE immunoblotting as described earlier. *C,* $p90^{RSK}$ activity. Cells were treated exactly as described in *A*, and the $p90^{RSK}$ was immunoprecipitated and assayed as described earlier. The $p90^{RSK}$ activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *Cont,* control.

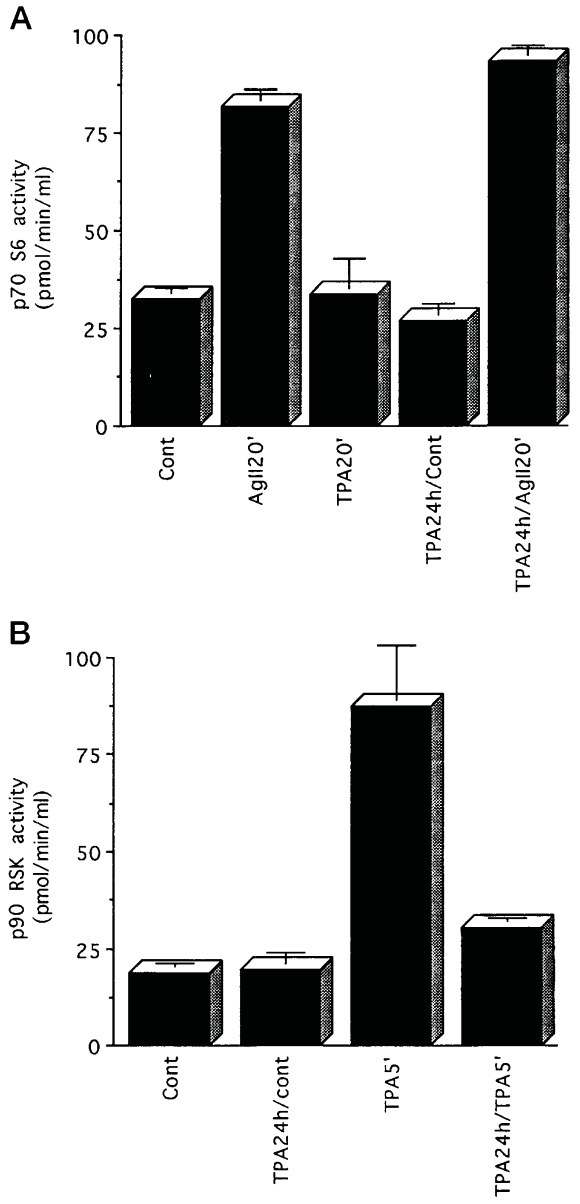

FIG. 6. **TPA activates p90^{RSK} but not p70^{S6K} in GN4 cells.** Serum-starved GN4 cells were stimulated with Ang II (*AgII,* 1 μM) or TPA (100 nM) for the amount of time indicated. In some experiments, cells were incubated with 1 μM TPA 24 h prior to collection. Cell lysates were prepared as described earlier and assayed for p70^{S6K} (*A*) or p90^{RSK} (*B*) activity. Kinase activity is plotted as pmol/min/ml, and the results represent the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 4$ experiments. *Cont,* control.

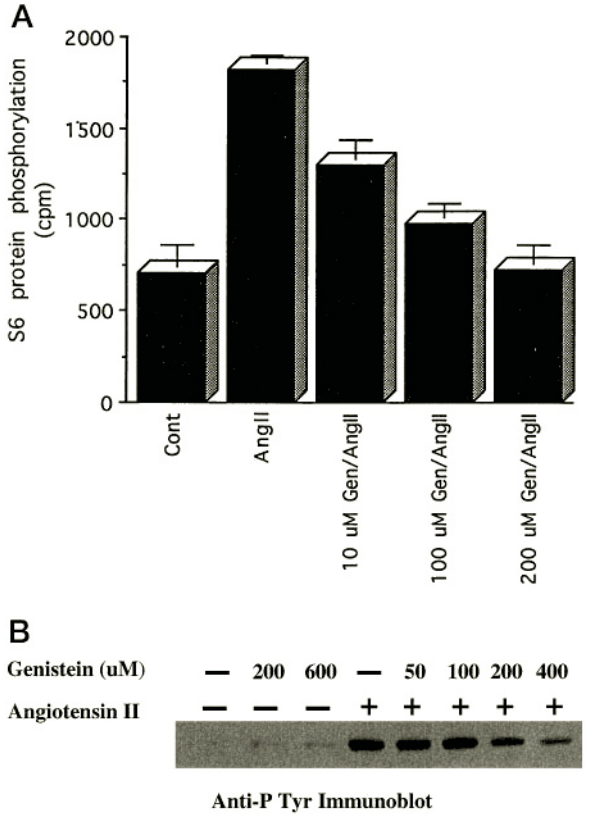

FIG. 7. **Genistein inhibits the activation of CADTK and p70^{S6K} by Ang II.** *A,* p70^{S6K} activity, serum-starved GN4 cells were incubated with Me_2SO (carrier for genistein) or genistein (*Gen*) for 15 min prior to the addition of Ang II (1 μM). After incubating cells with Ang II for 20 min, the cells were harvested, and the cell lysates were assayed for S6 kinase activity. The activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein and represents the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *Cont,* control. *B,* CADTK, GN4 cells grown in 6-cm plates were serum-starved and incubated with the concentrations of genistein indicated for 15 min. After incubation with genistein, the cells were treated with 1 μM Ang II for 90 s, and the cells were washed twice with PBS and harvested in RIPA buffer (1 ml). The amount of tyrosine autophosphorylation on CADTK was determined by immunoprecipitation with CADTK-specific antisera followed by PT66 antiphosphotyrosine immunoblotting as described under "Experimental Procedures."

thelial cells (4), cloned the cDNA, and raised specific antisera to the protein (5). We compared the ability of genistein to inhibit the activity of CADTK by assaying the tyrosine autophosphorylation of CADTK, as described under "Experimental Procedures." The amount of tyrosine autophosphorylation of this kinase appears to be proportional to the enzymatic activity.[2] As shown in Fig. 7*B*, incubating GN4 cells with concentrations of genistein from 50 to 400 μM led to a progressive decrease in the Ang II-stimulated CADTK tyrosine autophosphorylation. Similar concentrations of genistein were required to inhibit the tyrosine autophosphorylation of the EGF receptor in these cells. In comparison, the calcium-dependent tyrosine phosphorylation of an exogenous substrate (*e.g.* paxillin, a 68–70-kDa protein) tyrosine-phosphorylated by CADTK was inhibited by even lower concentrations of genistein (100–200 μM) than was the CADTK autophosphorylation.[3] The results with genistein implicate a tyrosine kinase in the activation of p70^{S6K} by calcium or Ang II, suggesting a role for CADTK.

The Stimulation of p70^{S6K} Correlates with Increased CADTK Activity—To further investigate whether CADTK was involved in the regulation of p70^{S6K}, we compared the activation of p70^{S6K} in cells known to exhibit different levels of Ang II- and thapsigargin-dependent CADTK activation. Previously we showed that transformed GN4 rat liver epithelial cells exhibit approximately 3–4 times more Ang II-stimulated tyrosine phosphorylation than the parental cell type from which it was derived (WB) (3). We have recently confirmed that GN4 cells exhibit approximately 5 times more Ang II-dependent activation and 2–3-fold more CADTK protein than WB cells (5). Treatment with Ang II or the ionophore A23187 resulted in a significantly larger activation of p70^{S6K} in GN4 cells compared with WB cells (Fig. 8*A*). Typically, the ability of Ang II or A23187 to activate p70^{S6K} was at least 2–3-fold higher in GN4 cells than in WB cells and was independent of the length of

[2] L. Xiong, unpublished observations.

[3] L. Xiong, unpublished data.

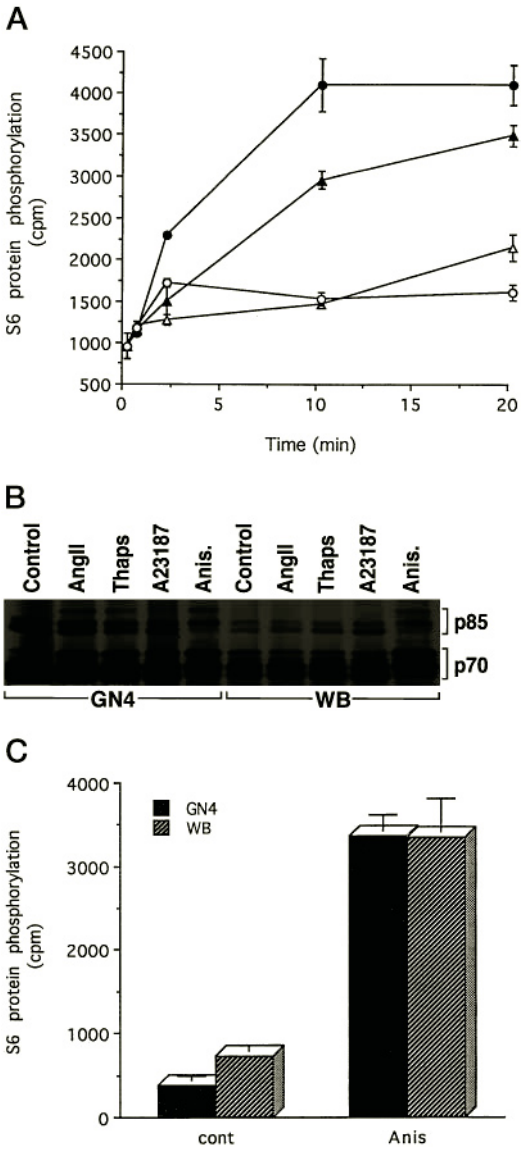

FIG. 8. **Increased expression of CADTK in GN4 cells correlates with increased activation of p70^{S6K} by calcium stimuli.** *A,* serum-starved GN4 cells were incubated with 1 μM A23187 (▲) or 1 μM Ang II (●), and serum-starved WB cells were treated with 1 μM A23187 (△) or 1 μM Ang II (○) for the lengths of time indicated. The cells were washed twice with PBS, and the cell lysates were assayed for p70^{S6K} activity. The activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein and represents the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 3$ experiments. *B,* GN4 and WB cells were stimulated with Ang II (1 μM), thapsigargin (*Thaps,* 2 μM), A23187 (1 μM), or anisomycin (*Anis.*, 10 μg/ml) for 20 min. Cell lysates were prepared, and the p70^{S6K} was detected by immunoprecipitation and immunoblotting as described earlier. *C,* GN4 (*solid bars*) and WB (*hatched bars*) cells were serum-starved and incubated with anisomycin (*Anis,* 10 μg/ml) for 20 min, after which the cells were harvested and assayed for p70^{S6K} activity as described earlier. The activity is plotted as radioactivity (cpm, ^{32}P) incorporated into the ribosomal S6 protein and represents the mean ± S.E. (*bars*) of duplicate samples. Data plotted are representative of $n = 2$ experiments. *cont,* control.

exposure to these compounds. Similarly, thapsigargin stimulated a similar 2–3-fold higher increase in S6 kinase activity in GN4 cells (data not shown).

To eliminate the possibility that the enhanced calcium-dependent activation was simply due to increased expression of the p70^{S6K} in GN4 cells, the amount of p70^{S6K} activity and protein was compared in the two cell lines by immunoblotting and activity assays as described earlier. As determined by immunoblotting, the amount of the p70^{S6K} protein and the nuclear form of this kinase (p85^{S6K}) (33) was comparable in the two cell types (Fig. 8*B*). Importantly, in GN4 cells, Ang II, thapsigargin, and A23187 stimulated a pronounced mobility shift of both the p70 and p85 S6 kinases; by contrast, much less mobility shift was observed in WB cells, in agreement with the S6 kinase activity assays. Furthermore, the protein synthesis inhibitor anisomycin (20) strongly stimulated the mobility shift (Fig. 8*B*) and activity of p70^{S6K} (and p85^{S6K}) equivalently in WB and GN4 cells (Fig. 8*C*). These results demonstrate that differences in p70^{S6K} or p85^{S6K} expression do not account for the enhanced activation by calcium in GN4 cells. Instead these results suggest that the increased expression of CADTK in GN4 cells facilitates the activation of p70^{S6K} by Ang II or calcium stimuli.

DISCUSSION

Ang II has numerous effects on rat liver epithelial cells. It is a weak mitogen, a response that is initiated via the AT_1 receptor, transduced by G_q protein stimulation of phospholipase C, and effected by serine, threonine, and tyrosine phosphorylation (1). Elevating intracellular calcium with thapsigargin or ionophore (A23187) mimics the Ang II-stimulated increase in protein tyrosine phosphorylation in GN4 cells (2), and our laboratory has recently isolated a likely candidate for the calcium-stimulated entity that regulates tyrosine phosphorylation, a novel CADTK. Incubating GN4 cells with Ang II or agonists that raise intracellular calcium (*i.e.* thapsigargin and A23187) activates CADTK (4), whereas incubation with the calcium chelator BAPTA-AM inhibits activation of this enzyme. The effect of calcium on CADTK is indirect; adding calcium or calcium and calmodulin to cell lysates does not activate CADTK, and thus the mechanism by which calcium and other signals regulate this enzyme remains to be established.

In addition to activating PKC, the calcium- and calmodulin-dependent protein kinase, and CADTK in GN4 cells, Ang II also activates MAPK and JNK and increases AP-1 binding (the latter can be accomplished in a PKC-independent manner) (10). We now demonstrate Ang II and calcium-dependent activation of p70^{S6K}. The challenge is to discern which of these multiple Ang II-dependent pathways are downstream of PKC, calcium and calmodulin, CADTK, or even G-protein $\beta\gamma$ subunits.

We began this process by purifying CADTK; peptide and cDNA cloning has identified this enzyme as the rat homologue of a novel human nonreceptor tyrosine kinase, PYK-2 (4–6). Using PC12 cells, PYK-2 was shown to be activated by elevating intracellular calcium or PKC activity. In these cells, PYK-2 stimulation increased MAPK activity, providing a potential mechanism for calcium-dependent regulation of MAPK in these and other cells (6, 52). In contrast, our studies in rat epithelial cells demonstrate that a calcium signal (*i.e.* thapsigargin and A23187) does not significantly activate MAPK and that Ang II-dependent MAPK activation is primarily a PKC-dependent process.[2] Instead, Ang II and a calcium signal (thapsigargin) substantially activate JNK in a calcium-dependent manner (10). In fact, the Ang II effect is PKC-independent and is amplified in cells depleted of PKC. JNK activation by Ang II and thapsigargin correlates with increased expression and tyrosine autophosphorylation of CADTK in our rat liver epithelial cells. We have shown that the chemically transformed GN4 cell line expresses more CADTK than the parental cell line (WB), and the activation of CADTK by Ang II is approximately 5-fold greater in confluent GN4 cells when compared with WB cells. Activation of JNK by Ang II and thapsigargin is also 5-fold higher in GN4 cells, whereas the total JNK activity induced by stress (*e.g.* anisomycin) is equal in WB and GN4 cells. Thus in GN4 cells, CADTK is not involved in MAPK activation but is likely to be a mediator of signaling to JNK.

Because some of the stimuli known to activate JNK also increase $p70^{S6K}$ activity in other cell types, (*i.e.* Ang II (11) and anisomycin (20)), we compared the ability of a calcium signal to regulate $p70^{S6K}$ in epithelial cells. In this study we find that like JNK (10), $p70^{S6K}$ is regulated by agonists that raise intracellular calcium, whereas neither $p90^{RSK}$ nor MAPK is affected by the calcium-activated pathway. This is contrasted to results in PC12 cells (6, 52) and suggests that the ability of calcium to regulate these signaling pathways is cell type-specific.

The role of intracellular calcium and Ang II (through its effects on intracellular calcium) in stimulating $p70^{S6K}$ has not been studied extensively. In one article, A23187 weakly activated $p70^{S6K}$ in Swiss 3T3 fibroblasts (53), a cell line that does not appear to contain CADTK.[2] Earlier, the work of Meier *et al.* (54) reported the activation of an S6 kinase in kidney epithelial cells in response to calcium stimuli (A23187), although the identity of this kinase was not established. It is clear that in this study we were measuring the calcium-dependent activation of $p70^{S6K}$ in cell lysates. First, although the cell lysate assay could detect $p90^{RSK}$ phosphorylation of the 40 S ribosomes, we have used a specific immune complex assay to show that there is little if any thapsigargin-dependent $p90^{RSK}$ activation. Second, all our assay data have been confirmed by immunoblot analysis of the $p70^{S6K}$ mobility shift, an assay that highly correlates with the activation. Third, rapamycin, which inhibits $p70^{S6K}$ but not $p90^{RSK}$ activation, abolished the Ang II- or thapsigargin-dependent activation of 40 S ribosome phosphorylation in cell lysates.

Importantly, our results suggest that most of the Ang II-dependent activation of $p70^{S6K}$ is calcium-dependent. Preincubation with BAPTA prevented the Ang II-dependent activation of $p70^{S6K}$ but not $p90^{RSK}$. Instead, $p90^{RSK}$ and MAPK were substantially activated by BAPTA, alone or in the presence of Ang II. At this point, the mechanism of the BAPTA effect is unclear; we can only speculate that calcium may regulate one or more phosphatases in the MAPK cascade or that BAPTA has a non-calcium-dependent effect on the MAPK pathway. We also cannot exclude that additional G-protein-coupled signals, *e.g.* $\beta\gamma$ subunit or other G-protein-dependent responses, contribute to the stimulation of $p70^{S6K}$ by Ang II. Recently the activation of $p70^{S6K}$ by receptors coupled to G_i has been described, supporting a potential role for $\beta\gamma$ subunits in the regulation of $p70^{S6K}$ (55).

In this study we also found that agonists that increase intracellular calcium (A23187, thapsigargin, and Ang II) in GN4 cells activate $p70^{S6K}$ following a rapid stimulation of CADTK. The cell-permeable chelator BAPTA-AM inhibited the activation of both CADTK and $p70^{S6K}$ by thapsigargin, A23187, or Ang II, consistent with a role for CADTK upstream of $p70^{S6K}$ in these cells. In further support of a role for CADTK, the calcium- and Ang II-dependent activation of $p70^{S6K}$ was prevented by genistein at concentrations similar to those required to inhibit the thapsigargin-dependent JNK activation and the thapsigargin- and Ang II-dependent tyrosine phosphorylation of CADTK substrates (*e.g.* paxillin) *in vivo*.[2] Other findings linking CADTK to the $p70^{S6K}$ pathway come from a comparison of $p70^{S6K}$ activity in WB and GN4 cells. The GN4 cell line expresses 2–3-fold greater CADTK and has 5-fold greater Ang-II-dependent CADTK and JNK activation (5). GN4 cells also exhibit 2–3-fold greater $p70^{S6K}$ activation in response to Ang II and thapsigargin. The failure of $p70^{S6K}$ to match the 5-fold difference in CADTK and JNK activation between GN4 and WB cells may well stem from the complex pathway to $p70^{S6K}$, which appears to involve regulation of at least two upstream kinase pathways (see below).

One possibility is that a calcium signal may be activating PI-3 kinase, since low concentrations of wortmannin (50 nM) inhibited the activation of $p70^{S6K}$ by A23187, thapsigargin, or Ang II. Numerous studies have suggested that PI-3 kinase is an upstream mediator of $p70^{S6K}$ activity (reviewed in Ref. 12), although much of the evidence for PI-3 kinase (including our study) has been obtained with the inhibitor wortmannin (48), the selectivity of which has been recently questioned (56). With the caveat of specificity, the activation of CADTK by Ang II or thapsigargin is not inhibited by wortmannin,[2] suggesting that PI-3 kinase is downstream or unrelated to the calcium-dependent activation of CADTK. Wortmannin also does not inhibit JNK activation, another pathway linked to the regulation of CADTK in GN4 cells.[2] Thus, if CADTK is a common activator of both $p70^{S6K}$ and JNK in GN4 cells, as our results suggest, at some point the signaling pathways diverge such that a wortmannin-sensitive step is specific to the $p70^{S6K}$ pathway.

Our results are also consistent with the calcium-dependent activation of another limb of the $p70^{S6K}$ pathway, the one that activates the rapamycin-sensitive kinase, *i.e.* FRAP/RAFT. Rapamycin inhibits the activity of the FRAP/RAFT kinases by binding to its cognate binding protein, FKB12, thus inhibiting $p70^{S6K}$ stimulation (16). However, like the PI-3 kinases, the role of FRAP/RAFT in regulating $p70^{S6K}$ is poorly understood, largely because substrates for these kinases remain to be identified. Again the calcium-dependent pathway to JNK and $p70^{S6K}$ is divergent in GN4 cells, since rapamycin blocks Ang II- and thapsigargin-dependent $p70^{S6K}$ activation but does not effect the stimulation of CADTK or JNK by these agonists.[2]

In addition to increasing intracellular calcium, Ang II also stimulates PKC activity (1, 5). Despite the fact that PKC has been shown to activate $p70^{S6K}$ in other cell types (57), our results do not support a role for PKC in the regulation of $p70^{S6K}$ in GN4 cells. Although Ang II stimulated $p70^{S6K}$ in a manner similar to thapsigargin or A23187, TPA alone did not increase $p70^{S6K}$ activity in GN4 cells, although MAPK and $p90^{RSK}$ were activated as expected.

TPA also increases CADTK activity in these cells, albeit less potently than Ang II and more slowly than thapsigargin. These results present a paradox, since our studies suggest that CADTK is upstream of $p70^{S6K}$, and yet TPA does not activate $p70^{S6K}$. We have found that a brief (5 min) TPA pretreatment inhibited the activation of $p70^{S6K}$ by Ang II or thapsigargin by approximately 40% in GN4 cells.[4] Similarly, an even more profound inhibitory effect of PKC has been found on the activation of JNK by calcium stimuli or Ang II in GN4 cells.[2] Taken together, these studies demonstrate that PKC alone is incapable of activating $p70^{S6K}$ and may inhibit the downstream calcium-dependent signals to both JNK and $p70^{S6K}$ in GN4 cells.

In summary, we have demonstrated a calcium-dependent pathway that results in substantial activation of $p70^{S6K}$. This calcium-dependent pathway has characteristics similar to to the calcium-dependent pathway to JNK that we have previously studied in this cell line (5, 10). Intriguingly, this calcium-dependent pathway does not activate MAPK or $p90^{RSK}$; instead these enzymes are activated in PKC-dependent manner. Considerable circumstantial evidence points to CADTK as an initial mediator of the calcium signal to both JNK and $p70^{S6K}$. However, the pathway to these enzymes clearly diverges at some point such that the calcium-dependent activation of JNK is insensitive to wortmannin and rapamycin, whereas the activation of $p70^{S6K}$ is completely inhibited by these compounds. The exact signaling downstream from CADTK and, in fact, unequivocal proof of CADTK involvement must await further studies.

[4] L. M. Graves, unpublished observations.

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