Activation of p70 S6 Kinase and erk-encoded Mitogen-activated Protein Kinases Is Resistant to High Cyclic Nucleotide Levels in Swiss 3T3 Fibroblasts*

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Treatment of Swiss mouse 3T3 fibroblasts with certain cyclic nucleotide phosphodiesterase inhibitors (theophylline, SQ 20,006, and MY-5445) prevents the activation of the M, 70,000 S6 kinase (p70S6k) induced by a variety of external stimuli. Concentrations giving half-maximal inhibition were 800, 50, and 25 μM, respectively. Western blot analysis and immunocomplex kinase assays showed that these compounds inhibit the phosphorylation and activation of p70S6k without affecting the erk-encoded mitogen-activated protein (MAP) kinases or the erk encoded S6 kinase (p90Rsk). A distinct collection of cAMP and cGMP agonists and analogues did not suppress p70S6k activation, indicating that 1) high intracellular cyclic nucleotide concentrations do not antagonize the p70S6k pathway and 2) phosphodiesterase inhibitors block p70S6k activation by a mechanism that is independent of cAMP or cGMP production. The effect of theophylline and SQ 20,006, but not MY-5445, on p70S6k signaling may be due in part to the inhibition of a phosphatidylinositol 3-kinase that acts upstream of p70S6k. Finally, in contrast to many other cell types, cAMP and cGMP were also found to have no inhibitory effect on the MAP kinase/p90Rsk signaling pathway in Swiss 3T3 fibroblasts.

Addition of mitogens to quiescent mammalian cells induces a signaling cascade that leads to the multiple phosphorylation of 40 S ribosomal protein S6 (1). S6 phosphorylation is thought to increase the rate of synthesis of certain proteins which are required for efficient G1 progression and whose mRNAs contain a polyuridylinate tract at the 5′ end (2). Two families of mitogen-stimulated S6 kinases have been identified: the M, 70,000 S6 kinases (p70S6k)2 (1, 3, 4) and the M, 90,000 ribosomal S6 kinase (p90Rsk) (5). Enzymes in both families are activated by phosphorylation of serinethreonine residues (6–8). p90Rsk is activated by mitogen-activated protein (MAP) kinases (9) and participates in a signaling network that includes ras, raf-1, and Mek1 (10). By contrast, p70S6k lies on a distinct pathway that does not appear to include MAP kinases (11).

Injection of antibodies that neutralize p70S6k activity (12) and use of the immunosuppressant rapamycin, which blocks the activation of the enzyme (13, 14), has suggested that p70S6k function during the G1 phase of the cell cycle is important for proliferation in some cell types. Three serines and one threonine clustered at the carboxyl terminus of p70S6k become phosphorylated in response to mitogen treatment (15). However, recent data have shown that deletion of the carboxyl terminus (16) or mutation of the four mitogen-induced phosphorylation sites to acidic residues yields p70S6k molecules which can still be activated by mitogens. Therefore, the contribution of these sites to enzyme activation remains unclear. In addition to the four mitogen-responsive phosphorylation sites, p70S6k contains other phosphates that turn over very slowly and that appear to be essential for enzyme activity. Rapamycin induces the dephosphorylation of these unmapped sites and therefore prevents the activation of p70S6k (17).

Little is known about the signaling components that function upstream of p70S6k. One approach to identify participants in the p70S6k pathway has been to study the mechanism of action of inhibitors of the pathway. For example, Kunz and co-workers (18) showed that rapamycin suppresses the growth of Saccharomyces cerevisiae by interacting with two gene products encoded by TOR1 and TOR2. Homologous proteins were subsequently found in higher eukaryotes (19). These proteins show significant homology to the catalytic subunit of mammalian phosphatidylinositol 3-kinase (Ptdlns 3-kinase) (20), which plays an important role in mitogenesis and other cellular responses (21, 22). It was subsequently shown that wortmannin and other specific inhibitors of mammalian Ptdlns 3-kinase also prevent p70S6k activation induced by a variety of agents (23–25). Together, these results suggested that Ptdlns 3-kinase or a related enzyme might be involved in the activation of p70S6k. This conclusion is supported by the recent observation that expression of a constitutively active Ptdlns 3-kinase leads to activation of p70S6k and phosphorylation of a novel site within the kinase catalytic domain (26).

A second possible class of inhibitors of the p70S6k pathway was suggested by work of Thomas and co-workers (27), who showed that pretreatment of Swiss mouse 3T3 fibroblasts with theophylline or SQ 20,006 blocked the serum-induced phosphorylation giving 50% inhibition; BSA, bovine serum albumin.

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rylation of S6. We demonstrate here that these two compounds block S6 phosphorylation by selectively inhibiting the activation of p70S6K. Theophylline and SQ 20,006 are best known as nonspecific cyclic nucleotide phosphodiesterase inhibitors (28, 29) that might raise the intracellular concentration of cAMP and cGMP, leading to the activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively). It has recently been shown that cAMP antagonizes p70S6K activation in T cells (30) and the MAP kinase/p90S6K pathway in a number of other cell types (31–35). However, we show that inhibition of p70S6K activation in Swiss 3T3 fibroblasts by theophylline and SQ 20,006 is independent of increased cyclic nucleotide concentrations or PKA activation. Finally, we find that cyclic nucleotides do not negatively regulate either the p70S6K or the MAP kinase/p90S6K pathways in this cell type.

**EXPERIMENTAL PROCEDURES**

**Materials—**Mouse epidermal growth factor (EGF) was purchased from Biomedical Technologies, Inc. and recombinant human platelet-derived growth factor (PDGF) was purchased from Boehringer Mannheim. Theophylline and prostaglandin E1 (PGE1) were from Serva. SQ 20,006 was a gift from Bristol-Myers Squibb. Bombesin, 3-isobutyl-1-methylxanthine (IBMX), cycloheximide, phosphor 12-myristate 13-acetate (PMA), Kemptide, 8-Br-cAMP, myelin basic protein, and a Protein inhibitor peptide (PKI) were from Sigma. Bovine insulin and A23187 were from Calbiochem. S9, 8-Bromoadenosine-3’-5’ cyclic monophosphorothioate (S9-8-BrcAMP), 8-Br-cGMP, S-nitroso-N-acetylpenicillamine (SNAP), and MY-544 were from Biolog. 32P-ATP (3000 Ci/mmol) was from Amersham Corp. Polyclonal antibodies to p90rsk and the M, 42,000 and 44,000 MAP kinases (p42MAPK and p44MAPK) were from Upstate Biotechnology, Inc. 4S ribosomal subunits were purified from rat liver (36). Tissue culture medium and fetal calf serum were purchased from Life Technologies, Inc.

**Cell Cultures and Extraction—**Swiss mouse 3T3 fibroblasts were maintain as described previously (4). Unless otherwise stated, all experiments were done on quiescent, contact-inhibited cells. To make extracts, the cell layers were washed twice with cold extraction buffer (20 mM Tris, 20 mM EGTA, 15 mM MgCl2, 40 mM 4-nitrophenyl phosphate (pNPP), 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5), the cells were scraped into 400 μl of extraction buffer, and extracts were centrifuged at 8000 x g for 15 min at 4°C. Supernatants were retained.

**Protein concentration** was determined by a Lowry assay (37).

**S6 Kinase and MAP Kinase Assays—**S6 Kinase and MAP kinase immunocomplexes were isolated from cell extracts by immunoprecipitation with antibody 122 against p42MAPK and PKA assay buffer (50 mM Tris, 10 mM MgCl2, 0.1% Triton X-100, and 0.25 mg/ml bovine serum albumin (BSA), pH 7.5) and assayed for S6 kinase activity as described previously (6), except reactions also contained 2 μM PKI. One unit is the amount of enzyme that incorporates 1 pmol of Pi into S6 per min.

For S6 kinase immunocomplex assays, extract supernatants were diluted into immunoprecipitation buffer (50 mM Tris, 1% Triton X-100, 50 mM NaCl, 20 mM NaF, 1 mM benzamidine, 5 mM EGTA, 10 mM PP3, 30 μg/ml leupeptin, 100 μM leupeptin, 1 μM pepstatin, 0.1% BSA, 0.1 mM DTT, and 0.1 mM PMSF, pH 7.2) and the solutions were incubated with antibodies to p70S6K for 2 h at 4°C. Protein A-agarose beads (Sigma) which had been preincubated with immunoprecipitation buffer were added, and the samples were incubated for 1 h at 4°C. The beads were washed twice with immunoprecipitation buffer and twice with S6 kinase assay buffer without DTT. S6 kinase assays were then performed as described above.

For MAP kinase immunocomplex assays, extract supernatants in immunoprecipitation buffer were incubated with antibody 122 against p42MAPK as described above. After washing with immunoprecipitation buffer, the protein A-agarose beads were washed twice with MAP kinase assay buffer (30 mM Tris, pH 8, 20 mM MgCl2, 2 mM MnCl2, 0.1% Triton X-100, and 0.1 mM DTT). MAP kinase assays were initiated by adding 15 μl of MAP kinase assay buffer containing 10 μM ATP, 2 μM PKI, 10 μg of myelin basic protein, and 0.33 μl of [γ-32P]ATP. After 30 min at 30°C the reactions were stopped by adding 10 μl of SDS sample buffer and heating at 95°C. The reactions were subjected to electrophoresis on SDS-20% polyacrylamide gels and autoradiography.

**PKA Assay—**Cells were washed twice with cold PBS and homogenized with 20 strokes in 500 μl of buffer containing 10 mM potassium phosphate, 100 mM KCl, 20 mM NaF, 0.5 mM theophylline, and 0.1 mM PKI. Homogenates were centrifuged at 10,000 x g for 10 min at 8000 x g, and supernatants were diluted 2:1 in PKA assay buffer (56 kinase assay buffer plus 50 mM P, pH 7). Protein kinase activity was determined in the presence or absence of 10 μM 8-Br-cAMP or 4 μM PKI. Diluted cell extract (10 μl) was added to 10 μl PKA assay buffer containing 100 μM ATP, 20 mM pNPP, 50 mM Kemptide and 0.1 μl [γ-32P]ATP. Following incubation for 10 min at 30°C, the reactions were stopped by addition of 10 μl of 10% glycerol and 10 μl of 7% trichloroacetic acid. After centrifugation the supernatants were pipetted onto Whatman P-81 paper. The papers were washed four times with cold 75 mM phosphoric acid, rinsed with ethanol, dried, and counted in a scintillation counter. PKA activity ratios were calculated as described previously (39).

**cAMP Assay—**Cells were washed twice with cold PBS and lysed in 0.1 mM HCl for 30 min, and the lysates were centrifuged at 4°C for 10 min at 8000 x g. cAMP was purified from the supernatants and radiomunoassays were performed as described in the instruction manual of the RIANEM cAMP RIA Kit.

**Polyclonal Antibodies to p70-S6K—**A cDNA fragment encoding amino acids 288–469 of rat p70S6K (3) was inserted into the BamHI and HindIII sites of the vector pETH-2a (40) and expressed in Escherichia coli. The recombinant protein, which contained 12 additional amino acids at its amino terminus including 6 histidines and 2 additional amino acids at the carboxyl terminus, was purified on Ni2+-nitrilotriacetic acid-agarose (Qiagen) under denaturing conditions (41). The protein was further purified on SDS-polyacrylamide gels and used to raise antisera in rabbits. Purified recombinant protein was also coupled to CNBr-activated Sepharose 4B (Pharmacia) and used to affinity purify the antibodies (42). The purified antibodies showed no cross-react with p90 on Western blots or in immunocomplex kinase assays.

**Immuno blot—**Proteins (8 μg) in extract supernatants were resolved on SDS-15% polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked in 3% BSA for 1 h and incubated for 2–15 h in PBS, 0.5% Tween 20 containing purified antibody to p70S6K (1:1000 dilution), MAP kinase R2 antibody (recognizes p42MAPK and p44MAPK; 1:1000), or p90rsk antibody (1:1000). Membranes were washed several times and further incubated with anti-rabbit horseradish peroxidase-linked whole antibodies from donkey (1:5000; American Qualex Corp) for 1 h. Specific signals were detected with the ECL (Amersham).

**PtdIns 3-kinase Assays—**The catalytic (p110α) and regulatory (p85α) subunits of PtdIns 3-kinase were expressed in insect cells using the baculovirus system and partially purified by ion exchange and heparin chromatography (43). Lipid kinase assays were performed using PtdIns 4,5-bisphosphate as substrate as described elsewhere (43).

Anion Exchange Chromatography—Cells treated as described in the text were washed twice with cold buffer A (25 mM Tris, 40 mM pNP2, 2 mM EGTA, 1 mM DTT, and 1 mM benzamidine, pH 7.5) containing 0.1% PMSF, scaped to 900 μl of plate of buffer A containing PMSF and 0.1% Triton X-100 and homogenized with five strokes in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 4°C for 10 min at 8000 x g, and the supernatant was applied to a 0.5-mL mono Q column (Pharmacia Biotech Inc.) at a flow rate of 0.5 ml/min. After the column was washed with 2 ml of buffer B plus 0.1% Triton X-100, bound material was eluted with a 10-ml linear gradient from 0 to 0.5 M NaCl in buffer A plus 0.1% Triton X-100. Fractions (0.5 ml) were collected and assayed for S6 kinase activity.

**RESULTS**

Inhibition of S6 Kinase Activation by Theophylline and SQ 20,006—It was shown earlier that high concentrations of the nonspecific phosphodiesterase inhibitors theophylline and SQ

3. C. Petritsch, H. M. L. Edelmans, and L. M. Ballou, unpublished observations.
20,006 block the serum-induced phosphorylation of S6 in intact fibroblasts (27). This effect could be mediated by inhibition of an S6 kinase or activation of an S6 phosphatase. To test the first possibility, quiescent fibroblasts were pretreated with or without theophylline or SQ 20,006 and then EGF was added to stimulate S6 kinase. In control cells S6 kinase was maximally activated after 10 min of EGF treatment and then the activity slowly decreased (Fig. 1A). Pretreatment with theophylline or SQ 20,006 lowered the basal level of S6 kinase activity in unstimulated cells and greatly reduced the EGF-induced activation of the enzyme (Fig. 1A). Pretreatment with theophylline or SQ 20,006 also decreased the phosphorylation of S6 in vivo by causing the inhibition of an S6 kinase.

Selective Inhibition of p70 S6 Kinase Activation by Theophylline and SQ 20,006—Mouse fibroblasts contain both p90rsk and p70S6k (44). The majority of S6 kinase activity measured in cell extracts using 40 S ribosomal subunits as a substrate is contributed by p70S6k. To determine which of these kinases is sensitive to theophylline and SQ 20,006, p90rsk and p70S6k in fibroblast extracts were assayed after separating the two enzymes on an anion exchange column (Fig. 3A). The identity of p90rsk and p70S6k was confirmed by immunocomplex kinase assays using antibodies specific for each enzyme. Untreated quiescent cells showed a low level of S6 kinase activity, all of which appeared in a peak corresponding to p70S6k (Fig. 3A, 3B).
fraction 15). Treatment with SQ 20,006 almost completely abolished this basal activity. When control cells were exposed to EGF for a short time to maximally activate p90S6k (see Fig. 6C) a small peak of activity corresponding to this enzyme appeared in fraction 7. p70S6k was activated to a much larger extent (Fig. 3A, open circles). Pretreatment of cells with SQ 20,006 almost completely blocked the EGF-induced activation of p70S6k but did not inhibit p90S6k (Fig. 3A, closed circles). Virtually identical results were obtained with theophylline. Immunocomplex kinase assays of p70S6k also showed that this enzyme could no longer be activated by EGF in cells pretreated with theophylline or SQ 20,006 (Fig. 3B, upper panel, lanes 1–4). Thus, the effect of SQ 20,006 and theophylline seen in extracts (Fig. 1A) is due to the selective inhibition of p70S6k.

All evidence obtained so far indicates that p70S6k is activated by phosphorylation (7, 15, 17, 26). Therefore, theophylline and SQ 20,006 might reduce p70S6k activity either by affecting its phosphorylation or by stimulating the degradation of the enzyme. To distinguish between these two possibilities, we examined the protein levels and phosphorylation state of p70S6k on immunoblots. p70S6k migrates differently in SDS-polycrylamide gels depending on its phosphorylation state, with the highly phosphorylated and active form migrating more slowly than the dephosphorylated, inactive enzyme (7). In resting cells the kinase was present mainly as hypophosphorylated species and kinase activity in immunoprecipitates was low (Fig. 3B, lane 1). After EGF treatment p70S6k activity increased and only the most highly phosphorylated forms were detected on the immunoblot (Fig. 3B, lanes 3 and 4). These data demonstrate that theophylline and SQ 20,006 either prevent the EGF-induced phosphorylation of p70S6k or stimulate its dephosphorylation. In addition, no change in p70S6k protein levels was seen after treatment with theophylline or SQ 20,006 (Fig. 3B, lower panel), indicating that these agents do not induce the degradation of the enzyme.

p70S6k Activation Is Resistant to High Levels of cAMP and PKA Activity—Since theophylline and SQ 20,006 act as cyclic nucleotide phosphodiesterase inhibitors in vitro (28, 29), the regulation of p70S6k by these two compounds in intact cells might be mediated by cAMP and PKA. However, cAMP and PKA did not inhibit p70S6k activity directly in an in vitro assay. To determine whether high cAMP levels or PKA activity antagonize the p70S6k pathway in vivo, several specific cAMP agonists and analogues were tested for the ability to block S6 kinase activation. The compounds tested included PGE1, a hormone that activates adenylyl cyclase through a specific receptor, and 8-Br-cAMP, which are cell-permeant, hydrolysis-resistant cAMP analogues. To confirm these results. First, cAMP and PKA do not antagonize the p70S6k activation pathway in fibroblasts. Second, theophylline and SQ 20,006 block p70S6k signaling by a mechanism that is independent of cAMP or PKA. And third, the target of theophylline seems to be a common regulatory element in p70S6k signaling pathways induced by different agonists.

Effect of cAMP on p70S6k Activation in Other Cell Types—In contrast to our results in fibroblasts (Fig. 3B and Table I), cAMP has been reported to block the activation of p70S6k in T cells (30). T cells are unlike Swiss fibroblasts in that they undergo growth arrest in response to high intracellular cAMP concentrations (45). We therefore determined if an inhibitory effect of cAMP on p70S6k also occurs in two additional cell lines whose proliferation is sensitive to cAMP (38, 46). Cycling wild-type S49 mouse lymphoma cells (38) exhibited a relatively high level of S6 kinase activity that was not significantly reduced in the presence of 8-Br-cAMP (Fig. 4). Similar results were obtained with cycling Swiss fibroblasts (Fig. 4). In addition, the activation of S6 kinase in BAC-1 macrophages (46) induced by colony-stimulating factor-1 was not decreased but rather augmented by 8-Br-cAMP (Fig. 4). Chromatography of cell extract supernatants on a Mono Q column (see Fig. 3A) confirmed that the major S6 kinase activity in lymphocytes and macrophages is contributed by p70S6k. Thus, p70S6k can be activated in the presence of high intracellular cAMP concentrations in some cell types whose proliferation is arrested by cAMP.

Activation of p70S6k Is Resistant to High cGMP Levels—Theophylline and SQ 20,006 also inhibit cGMP-specific phosphodiesterases (28, 29) and by this mechanism may increase cGMP levels and activate PKG. We therefore tested specific cGMP agonists for the ability to inhibit p70S6k activation. Myo-5445, an inhibitor of cGMP-specific phosphodiesterases, completely blocked the activation of p70S6k by EGF (Fig. 1C) with an IC50 of 25 μM (Fig. 2). By contrast, SNAP, an activator of cGMP-dependent protein kinase, enhanced p70S6k phosphorylation in response to 5 μM cGMP (Fig. 2A). Thus, p70S6k activation is insensitive to cGMP, which indicates that p70S6k and PKG are activated by different mechanisms and that these two signaling pathways are independently regulated.

| TABLE I  |
|----------|
| **Effect of cAMP agonists or cAMP analogues on PKA activity and cAMP levels** |
| **Fibroblasts were treated with the indicated agents and extract supernatants were assayed for PKA activity and cAMP levels (see “Experimental Procedures.”)** |
| **Addition** | **PKA activity** | **cAMP** |
| % | | |
| Control | 19 | 0.8 |
| 5 mM EGF, 15 min | 8 | 0.5 |
| 5 mM theophylline, 30 min | 22 | 0.9 |
| 1 mM SQ 20,006, 30 min | 21 | ND |
| 300 μM PGE1, 15 min | 100 | 56 |
| 500 μM Sp-8-Br-cAMPS, 20 min | 78 | ND |
| 500 μM 8-Br-cAMP, 20 min | 87 | ND |
| **ND**, not determined. | | |

1. 100% PKA activity is the value obtained when assays were performed in the presence of 8-Br-cAMP.
2. Picomoles per 1 × 10^6 cells.
3. ND, not determined.
Vitro—A variety of evidence suggests that a PtdIns 3-kinase
function upstream of p70S6k.

Theophylline and SQ 20,006 inhibit PtdIns 3-kinase in
vitro—A variety of evidence suggests that a PtdIns 3-kinase
can be involved in the activation of p70<sup>S6k</sup> (23–26). We there-
fore tested whether the three inhibitors of p70<sup>S6k</sup> activation
identified here have an effect on PtdIns 3-kinase activity
(see "Experimental Procedures"). S6 kinase activity in control cells (+ PBS) was 5
units/mg.

![Image](https://i.imgur.com/3Q5Q5Q.png)

**Fig. 4. Effect of 8-Br-cAMP on p70<sup>S6k</sup> activity in different cell
types.** Cycling S49 lymphoma cells or Swiss fibroblasts were treated for
30 min with PBS (black bars) or 500 μM 8-Br-cAMP (gray bars). BAC-1
macrophages were co-treated for 15 min with 24,000 units/ml of colony-
stimulating factor-1 in the presence (gray bars) or absence (black bars)
of 500 μM 8-Br-cAMP. Extract supernatants were prepared and assayed
for S6 kinase activity (see "Experimental Procedures").

![Image](https://i.imgur.com/5Q5Q5Q.png)

**Fig. 5. Dose-response for inhibition of PtdIns 3-kinase activity.** Partially purified p110α-p85β heterodimers were incubated with increasing amounts of theophylline (●), SQ 20,006 (□) or MY-5445 (○) and assayed for lipid kinase activity (see "Experimental Procedures").

Guanylyl cyclase, and 8-Br-cGMP showed little effect on p70<sup>S6k</sup>
activity (Fig. 1C). Therefore, no correlation between increased
cGMP levels and suppression of the p70<sup>S6k</sup> activation pathway
was evident. Immunocomplex kinase assays and Western anal-
ysis confirmed that inhibition of the EGFR-induced activation of
p70<sup>S6k</sup> by MY-5445 was not mimicked by 8-Br-cGMP (Fig. 3B,
lanes 8 and 9). In addition, 8-Br-cGMP did not reduce the
stimulation of p70<sup>S6k</sup> in response to other agonists (Table II).

These results indicate that cGMP does not negatively regulate
p70<sup>S6k</sup>. Theophylline and SQ 20,006 inhibit PtdIns 3-kinase in
vitro—A variety of evidence suggests that a PtdIns 3-kinase
can be involved in the activation of p70<sup>S6k</sup> (23–26). We there-
fore tested whether the three inhibitors of p70<sup>S6k</sup> activation
identified here have an effect on PtdIns 3-kinase activity
in vitro. Dose-response experiments showed that theophylline
and SQ 20,006 significantly inhibited lipid kinase activity in
vitro, with IC<sub>50</sub> values of 810 μM and 183 μM, respectively (Fig.
5). MY-5445 did not inhibit PtdIns 3-kinase activity at concen-
trations up to 1.25 mM (Fig. 5). Thus, one in vivo target of
theophylline and SQ 20,006 might be a PtdIns 3-kinase that
functions upstream of p70<sup>S6k</sup>.

No inhibition of MAP Kinase Activation by Cyclic Nucleotide
Reagents—It has been reported that cAMP antagonizes the activation of MAP kinases in a number of different cell types
(31–34). We therefore tested whether cAMP has a similar effect
in Swiss fibroblasts. To determine the conditions under which
MAP kinases become fully activated, a time course of kinase
activation was visualized on Western blots probed with anti-
odies that recognize two erk-encoded MAP kinases referred to
as p42<sup>MAPK</sup> and p44<sup>MAPK</sup>. The two MAP kinases were fully
activated 2.5 min after addition of EGF, as indicated by their
shift to a slower mobility due to phosphorylation (Fig. 6A). The
kinases remained maximally active for up to 5 min and then
dephosphorylated species appeared after 10 min (Fig. 6A). Sub-
sequent experiments were done using extracts from fibroblasts
stimulated for 5 min with EGF.

To directly examine the effect of cyclic nucleotide reagents on
MAP kinase activity, the erk-2-encoded p42<sup>MAPK</sup> was precipi-
tated with a specific polyclonal antibody and assayed in immu-
nocomplexes. These assays showed that p42<sup>MAPK</sup> was strongly
activated by EGF (Fig. 6B, upper panel, lanes 1 and 2). This
response was still fully intact in cells pretreated with cAMP
analogues or PGE<sub>1</sub> (Fig. 6B, upper panel, lanes 5–7). p42<sup>MAPK</sup>
was also activated normally in cells pretreated with 8-Br-
cGMP (Fig. 6B, upper panel, lane 8). In contrast to the results
obtained with p70<sup>S6k</sup> (Figs. 1 and 3B), pretreatment of cells
with theophylline, SQ 20,006, or MY-5445 did not block the
activation of p42<sup>MAPK</sup> by EGF (Fig. 6B, upper panel, lanes 3, 4,
and 9). Thus, these compounds do not disrupt all EGF receptor-
mediated responses. We also tested whether MAP kinase acti-
vation induced by insulin or PDGF is affected by cAMP. Al-
though these two growth factors were relatively poor activators
of p42<sup>MAPK</sup> at these concentrations, it was evident that addi-
tion of 8-Br-cAMP had no inhibitory effect (Fig. 6B, upper
panel, lanes 10–13). Examination of MAP kinases in these cell
e xtracts on Western blots indicated that the activation of
p44<sup>MAPK</sup>, like p42<sup>MAPK</sup>, was also resistant to high cyclic nu-
cleotide levels and phosphodiesterase inhibitors (Fig. 6B, lower
panel). In addition, the growth factor-induced mobility shift of
p90<sup>SK</sup> was not affected by these reagents (Fig. 6C); these
results confirm and extend the finding in Fig. 3A. Thus, in Swiss
3T3 fibroblasts activation of the erk-encoded MAP kinases and
p90<sup>SK</sup> is not antagonized by cAMP or cGMP.

**DISCUSSION**

It was reported earlier that treatment of Swiss mouse 3T3
fibroblasts with the nonselective phosphodiesterase inhibitors
theophylline and SQ 20,006 blocks the mitogen-induced phos-
phorylation of ribosomal protein S6 (27). We show here that
these two compounds mediate this effect by inhibiting the
activation of p70<sup>S6k</sup> (Fig. 3). Theophylline and SQ 20,006 did
not act as general kinase inhibitors or disrupt all receptor-
mediated responses, as shown by the fact that activation of

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**Table II**

Inhibition of S6 kinase activation by theophylline and
cyclic nucleotide analogues

| Addition          | S6 kinase activity |
|-------------------|--------------------|
|                   | PBS | Theophylline 8-Br-cAMP | 8-Br-cGMP |
|                   | fold increase |
| Control            | 1.0 | 0.4 | 1.1 | 1.1 |
| 1 μM Insulin, 15 min | 2.4 | 0.6 | 2.2 | 2.7 |
| 100 μM cycloheximide, 30 min | 4.0 | 0.4 | 5.2 | 4.9 |
| 50 μM A23187, 40 min | 4.0 | 1.0 | 4.1 | 4.1 |
| 10 μM bombesin, 30 min | 5.2 | 1.4 | 5.3 | 6.0 |
| 1 μM PMA, 40 min | 6.8 | 1.6 | 6.7 | 6.8 |
| 384 nM PDGF, 20 min | 8.2 | 2.4 | 9.3 | 8.7 |
| 5 μM EGF, 10 min | 10.0 | 3.2 | 9.6 | 10.7 |
with the observation made earlier that PGE₁ does not inhibit serum-induced S6 phosphorylation in vivo (27). Finally, work by others has shown that cAMP/PKA promotes the proliferation of Swiss mouse 3T3 fibroblasts (48); therefore, this pathway would not be expected to interfere with the activation of p70S6k, which is also required for efficient cell cycle progression in this cell type (12, 13).

In contrast to our results, Monfar et al. (30) recently reported that cAMP inhibits the interleukin-2-mediated activation of p70S6k in CTL-L-20T cells, in part by antagonizing the activity of PtdIns 3-kinase. This discrepancy could be due to cell type or agonist specificity. Unlike Swiss fibroblasts, T cells arrest in the G₂ phase of the cell cycle in response to high cAMP concentrations (45) or rapamycin treatment (49). Since rapamycin inhibits interleukin-2-mediated p70S6k activation (14), it seems plausible that the p70S6k pathway could also be the target for a cAMP-dependent cell cycle block in T cells. On the other hand, Monfar et al. (30) used forskolin/IBMX as a cAMP agonist, raising the possibility that p70S6k activation in a T cell lymphoma line (Fig. 4) also suggests that the sensitivity of T cell p70S6k to forskolin/IBMX might be independent of cAMP. Macrophages also arrest in the G₂ phase of the cell cycle in response to high cAMP levels or exposure to rapamycin (46). We found that 8-Br-CAMP enhances rather than inhibits the activation of p70S6k by colony-stimulating factor-1 in these cells (Fig. 4).

Since theophylline and SQ 20,006 also inhibit cGMP-specific phosphodiesterases in vitro (28, 29), we explored the possibility that cGMP and PKG might be involved in the inhibition of p70S6k activation. Although MY-5445, a selective inhibitor of cGMP-specific phosphodiesterases, strongly blocked the activation of p70S6k, 8-Br-cGMP and SNAP, a guanylyl cyclase activator, had no effect (Fig. 1C and Table II). These results strongly suggest that inhibition of p70S6k by MY-5445 is not a consequence of increased cGMP production or PKG activity.

Having ruled out the possibility that cAMP/PKA or cGMP play a role in the theophylline-induced p70S6k inhibition, we searched for alternative mechanisms that might mediate this response. PtdIns 3-kinase, which has been proposed to act as a positive upstream regulator of p70S6k (23–26), seemed to be a likely target. Indeed, we show here that theophylline and SQ 20,006, but not MY-5445, also inhibit PtdIns 3-kinase activity in vitro (Fig. 5). However, inhibition of PtdIns 3-kinase cannot be the only reason why theophylline blocks the p70S6k pathway. We showed earlier that wortmannin, a specific inhibitor of PtdIns 3-kinase, is a poor inhibitor of bombesin- and PMA-induced p70S6k activation (25). We concluded from these studies that some pathways leading to p70S6k activation are independent of PtdIns 3-kinase (25). By contrast, theophylline strongly inhibited the p70S6k response to all of the agonists listed in Table II, including bombesin and PMA. These data suggest that theophylline might act on another target in addition to PtdIns 3-kinase. This target appears to be a regulatory element that functions either independently or in parallel to other pathways (such as a p70S6k phosphatase) or in every pathway (such as a p70S6k kinase).

Theophylline has several other known cellular effects that could play a role in its ability to inhibit the activation of p70S6k. Theophylline inhibits the activation of cAMP phosphodiesterases (47). Theophylline binds to cell-permeant cAMP analogues and PGE₁ does not block the stimulation of p70S6k induced by EGF (Fig. 1B) or other agonists (Table II), even though PKA was strongly activated (Table I). These results are consistent
to a structural similarity to ATP (52). This property may account for the ability of theophylline to inhibit PtdIns 3-kinase activity in vitro (Fig. 5). Therefore, theophylline may inhibit the p70Sk pathway by targeting an upstream kinase that is required for a response to all agonists tested so far. Since no such upstream component has been identified yet, this hypothesis cannot be tested directly. SQ 20,006 and MY-5445 bear some structural resemblance to theophylline and may act in a similar manner. Identification or design of specific and potent inhibitors of p70Sk activation, based on the compounds we identified here, could lead to the isolation of upstream activators that function in this pathway.

We show here that the activation of p42MAPK/p44MAPK and p90Sr kinase in Swiss fibroblasts is also resistant to high cAMP and cGMP levels (Fig. 6). MAP kinase activation in PC12 cells is similarly resistant to high cAMP levels (31). By contrast, negative regulation of the MAP kinase/p90sk cascade by cAMP has been observed in Xenopus oocytes, Rat1 cells, smooth muscle cells, CHO cells, and adipocytes stimulated with a variety of agonists (32–35). In some of these cell types high cAMP levels also antagonize cell proliferation (33, 34) or meiotic cell division (35). Several different mechanisms may contribute to inhibition of MAP kinase activation, including a reduced activation of Raf-1 due to phosphorylation by PKA (34).

Our results, together with those from other laboratories, indicate that cyclic nucleotide-dependent signaling may interact in a variety of ways with the p70Sk and MAP kinase/p90Sk pathways. These interactions may be important for generating cell type-specific responses to identical physiological conditions. Identification of regulatory components in the p70Sk pathway will allow us to further elucidate the mechanisms of cell type-specific control.

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