Attenuation of Mammalian Target of Rapamycin Activity by Increased cAMP in 3T3-L1 Adipocytes*

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Pamela H. Scott‡ and John C. Lawrence, Jr.§¶

From the Departments of ¶Pharmacology and §§Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Incubating 3T3-L1 adipocytes with forskolin, which increases intracellular cAMP by activating adenylyl cyclase, mimicked rapamycin by attenuating the effect of insulin on stimulating the phosphorylation of four (S/T)/P sites in PHAS-I, a downstream target of the mammalian target of rapamycin (mTOR) signaling pathway. To investigate the hypothesis that increasing cAMP inhibits mTOR, the protein kinase activity of mTOR was measured in an immune complex assay with recombinant PHAS-I as substrate. Both forskolin and 8-(4-chlorophenylthio)adenosine 3’-5’-monophosphate (CPT-cAMP) prevented the activation of mTOR by insulin in adipocytes, but neither agent affected mTOR activity when added directly to the immunopurified protein. In contrast, the cAMP phosphodiesterase inhibitor, theophylline, inhibited mTOR activity not only when added to intact adipocytes but also when added to immunopurified mTOR in vitro, demonstrating that certain methylxanthines are able to inhibit mTOR independently of increasing cAMP. Forskolin and CPT-cAMP blocked the effect of insulin on increasing mTOR phosphorylation, which was assessed using mTAb1, an antibody whose binding is inhibited by phosphorylation of mTOR. Although the mTAb1 epitope contains a consensus site for protein kinase B, neither agent inhibited the activation of protein kinase B produced by insulin. These findings support the interpretation that increasing cAMP attenuates the effects of insulin on PHAS-I, p70^{s6k}, and other downstream targets of the mTOR signaling pathway by inhibiting the phosphorylation and activation of mTOR.

Translation of certain classes of mRNA in mammalian cells is regulated by a signaling pathway containing mTOR,† the mammalian target of rapamycin (1, 2). mTOR is the counterpart of Tor1p and Tor2p, two proteins required for cell cycle progression in Saccharomyces cerevisiae. Like the yeast proteins, mTOR contains a high affinity binding site for rapamycin-FKBP12. The function of mTOR in cells is potently inhibited by rapamycin, which has proven to be a useful pharmacological tool for identifying downstream elements in the mTOR signaling pathway. Rapamycin attenuates the phosphorylation of p70^{s6k} and PHAS-I that occurs in response to insulin (3, 4), and overexpression of mTOR increases the phosphorylation of both proteins (5, 6). These and other findings have established that mTOR controls these two regulators of mRNA translation.

p70^{s6k} phosphorylates ribosomal protein S6 and increases translation of mRNAs having the polypyrimidine tract (TOP) motif (7). The activation of p70^{s6k} is a function of a complex pattern of phosphorylation mediated by three or more protein kinases that phosphorylate at least 10 sites (8, 9). The rapamycin-sensitive sites, which by inference are those regulated by mTOR, include three in which the phosphorylated Ser/Thr is flanked by aromatic residues and one which fits a (Ser/Thr)-Pro motif (10). Nonphosphorylated PHAS-I binds tightly to eIF4E (11, 12), the mRNA cap-binding protein, and prevents eIF4E from interacting with eIF4G (13). Phosphorylation of PHAS-I leads to the dissociation of the PHAS-eIF4E complex (11, 12). This allows eIF4E to bind eIF4G to generate the complex that is needed for the efficient binding and/or scanning by the 40 S ribosomal subunit. In rat adipocytes, five (Ser/Thr)-Pro sites in PHAS-I are phosphorylated in response to insulin (14). Rapamycin attenuates, but does not abolish, the effects of insulin on the phosphorylation of these sites. The failure of rapamycin to inhibit fully the effect of insulin supports the view that an mTOR-independent pathway also contributes to the control of PHAS-I (15).

Significant progress has been made in understanding mechanisms involved in mTOR signaling. mTOR contains an essential COOH-terminal catalytic domain that is homologous to the catalytic subunit of PI 3-kinase (3, 4), and overexpression of mTOR increases the phosphorylation of both proteins (5, 6). These and other findings have established that mTOR controls these two regulators of mRNA translation.

† The abbreviations used are: mTOR, mammalian target of rapamycin; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3’-5’-monophosphate; eIF4E, eukaryotic initiation factor 4E; eIF4G, eukaryotic initiation factor 4G; FKBP12, FK506-binding protein of Mr = 12,000; HPLC, high performance liquid chromatography; MAP kinase, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PHAS-I, an eIF4E-binding protein also known as 4E-BP1; PI 3-kinase, phosphatidylinositol 3-kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; p70^{s6k}, the Mr ~ 70,000 ribosomal S6 protein kinase; TORK, mTOR kinase.

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, Box 448 Health Science Center, University of Virginia, Charlottesville, VA 22908. Tel.: 804-924-1584; Fax: 804-982-3575; E-mail: jcl3p@virginia.edu.

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34496
with phosphatase restored binding (18). The effects of insulin on mTOR in 3T3-L1 adipocytes were abolished by the PI 3-kinase inhibitor, wortmannin. Moreover, selectively activat-ing PKB with tamoxifen in MER-Akt cells, which express a PKB-estrogen receptor fusion protein that is rapidly activated when tamoxifen binds (19), mimicked insulin by increasing both the phosphorylation and activity of mTOR (18). These findings indicate that PI 3-kinase and PKB are upstream regulators of mTOR.

Pharmacological or genetic disruption of mTOR function may have striking inhibitory effects on mRNA translation and cell proliferation (1, 2). Although inhibition of mTOR activity is clearly a potential mechanism for regulation of cellular function, inactivation of mTOR by physiological regulators has not been described. Increasing cAMP has been shown to decrease the phosphorylation of PHAS-I and p70S6K in certain cell types (20–23). In this report, we present evidence that these effects of cAMP result from inactivation of mTOR.

EXPERIMENTAL PROCEDURES

Cell Culture and Incubations—3T3-L1 adipocytes were cultured in 10-cm dishes and used in experiments 8–10 days after plating the differentiation medium (3). Except for the 32P-labeling experiments (described in the legend to Fig. 1), cells were incubated at 37 °C for 2.5 h in HEPES-buffered saline (145 mM NaCl, 5.4 mM KCl, 1.4 mM CaCl2, 1.4 mM MgCl2, 0.1 mM NaF, 5 mM glucose, 0.5% bovine serum albumin, and 10 mM Na-HEPES, pH 7.4) and then incubated with insulin and other agents as indicated. To terminate the incubation, the medium was aspirated, and the cells were rinsed and homogenized in 1 ml of Homogenization Buffer, which contained 50 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 0.1 mM microcinyst-L, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM KF, and 50 mM β-glycerophosphate, pH 7.4. Homogenates were centrifuged at 10,000 × g for 20 min, and the supernatants were retained for analyses. The protein content was determined by using bicinchoninic acid (24).

Immunoprecipitations—Antibodies to PHAS-I (25) and mTOR (16) were described previously. To generate antibodies to PKB, a peptide (CVDSDERRHFPPFSYASSTA) having a sequence identical to that of the last 21 amino acids in PKB was synthesized. Antibodies were purified by using columns containing resin prepared by coupling the peptide to Sulfolink beads (Pierce).

Immunoprecipitations were conducted using either non-immune IgG or the appropriate antibodies coupled to protein A-agarose (Life Technologies, Inc.). Briefly, antibodies (5 μg) were incubated for 1 h at 23 °C with protein-Aagarose beads (20 μl of a 1:1 suspension) in Tris-buffered saline (10 mM NaCl, and 50 mM Tris-HCl, pH 7.4). The beads were then washed three times (1 ml/wash) and suspended in TBS (20 μl).

Immunoprecipitations were conducted using either non-immune IgG or the appropriate antibodies coupled to protein A-agarose (Life Technologies, Inc.). Briefly, antibodies (5 μg) were incubated for 1 h at 23 °C with protein-Aagarose beads (20 μl of a 1:1 suspension) in Tris-buffered saline (10 mM NaCl, and 50 mM Tris-HCl, pH 7.4). The beads were then washed three times (1 ml/wash) and suspended in TBS (20 μl). Extract samples (800 μl) were incubated with beads for 90 min at 4 °C and then washed (1 ml buffer/wash). For PHAS-I immunoprecipitations, beads were washed twice with 1 ml of Buffer A (1 mM EDTA, 1 mM EGTA, 10 mM MgCl2, 10 mM HEPES, pH 7.4), twice with Buffer A plus 0.5 mM NaCl, and then twice with Buffer A. For mTOR immunoprecipitations, beads were washed twice with Buffer A, twice with Buffer A plus 0.5 mM NaCl, and then twice with Buffer B (50 mM NaCl, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 0.2 μM microcinyst-L, and 10 mM Na-HEPES, pH 7.4). For PKB immunoprecipitations, beads were washed twice with Buffer C (0.5 mM NaCl, 10% glycerol, 0.1% bovine serum albumin, 0.5% Triton X-100, and 25 mM HEPES, pH 7.4) and twice with Buffer D (10 mM MgCl2, 1 mM dithiothreitol, and 50 mM Tris-Cl, pH 7.5).

To elute PHAS-I from immune complexes, the beads were rinsed with 1 ml of H2O, suspended in 150 μl of 1% β-mercaptoethanol, 1 mM EDTA, and 10 mM Tris-Cl, pH 7.5, and incubated at 95 °C for 15 min. After centrifuging the samples at 10,000 × g for 10 min, the supernatant was retained for analyses of PHAS-I. When samples of mTOR or PKB were subjected to SDS-PAGE, proteins were eluted in SDS-sample buffer (27). When protein kinase activities were measured, beads were suspended in 50 μl of the appropriate reaction mixture.

Measurements of Protein Kinase Activity—mTOR and PKB activities were measured in the same extract samples. Extracts (800 μl) were first incubated with the mTOR antibody, mTAb2 (16), coupled to protein A-agarose. After centrifuging the samples, the supernatants were retained for measuring PKR activity (described below), and the pellets containing the mTOR immune complexes were washed as described above. To assay mTOR activity, the beads were suspended in 50 μl of mTOR Reaction Mix that contained the following: 50 mM NaCl, 1 mM dithiothreitol, 10 μM PKA inhibitory peptide (28), 0.2 mM microcinyst-L, 20 μg/ml [32P]Pi, 0.2 μM [γ-32P]ATP (100 Ci/mmol), 10 mM MgCl2, 50 mM β-glycerophosphate, and 0.1 M HEPES, pH 7.4. Supernatants remaining after removing the mTOR immune complexes were incubated with PKB antibodies coupled to protein A-agarose. The beads were then washed and suspended in a reaction mixture identical to that described previously (30), except that histone 2B was substituted for myelin basic protein. mTOR and PKB samples were incubated at 30 and 23 °C, respectively, before the reactions were terminated by adding SDS sample buffer.

Peptide Mapping of 32P-PHAS-I—Samples of PHAS-I that had been immunoprecipitated from 32P-labeled 3T3-L1 adipocytes were digested with lysyl endopeptidase C (Wako Pure Chemicals) and chymotrypsin (Boehringer Mannheim) before phosphopeptides were resolved by reverse phase-high performance liquid chromatography (HPLC) as described previously for phosphorylation site analysis of rat adipocyte PHAS-I (14).

SDS-PAGE and Immunoblotting—Samples were subjected to SDS-PAGE (27), and 32P-labeled proteins were detected by autoradiography of the dried gels. mTOR (16) and PHAS-I (11) were detected by immunoblotting.

RESULTS

Rapamycin and Forskolin Decrease Phosphorylation of (S/T)P Sites in PHAS-I—Both rapamycin and increasing cAMP promote dephosphorylation of PHAS-I in 3T3-L1 adipocytes (3, 21). As a step in determining whether rapamycin and cAMP affect a common upstream regulator of PHAS-I, experiments were conducted to determine whether forskolin, an agent that increases intracellular cAMP, affected the same sites in PHAS-I as rapamycin. Peptide mapping followed by amino acid sequence analyses was previously used to identify Thr36, Thr45, Ser44, Thr48, and Ser82 as sites of phosphorylation in PHAS-I in rat adipocytes (14). These five residues, as well as the sites for cleavage by the proteases used in the previous mapping studies (14), are present in 3T3-L1 PHAS-I (3, 25). Indeed, except for peptides containing Ser82, 3T3-L1 PHAS-I would be expected to yield fragments identical to those derived from rat PHAS-I. Thus, we were able to resolve phosphorylation sites in PHAS-I from 3T3-L1 adipocytes by using the strategies developed for analyses of the rat protein.

To label sites in PHAS-I, 3T3-L1 adipocytes were incubated in medium containing [32P]Pi. After incubating with the indicated additions, extracts of the cells were prepared. [32P]Pi-PHAS-I was then immunoprecipitated and digested with lysyl endopeptidase. The resulting phosphopeptides were subjected to reverse phase-HPLC (Fig. 1A). Three peaks, designated LE-P1, LE-P2, and LE-P3, eluted in the same position as peaks derived from rat PHAS-I (14). In view of the near identity of rat (25) and 3T3-L1 (3) PHAS-I proteins (Fig. 1C), it seems reasonable to assume that the peaks represent the phosphorylation sites identified previously (14). Thus, LE-P1 and LE-P2 are presumed to contain Thr36 and Ser84, respectively. Thr45 and Thr48 are recovered in a fragment eluting in LE-P3. These two sites may be resolved after digesting this fragment with chymotrypsin (14), and the peaks detected at CT-P1 and CT-P2 correspond to peptides containing Thr45 and Thr48, respectively (Fig. 1B). A relatively small third peak containing a peptide with phosphorylated Ser82 was previously detected after digesting LE-P3 from rat adipocyte PHAS-I with chymotrypsin (14). This peak was absent in samples derived from the 3T3-L1 protein. Thus, Ser82 does not seem to be phosphorylated to a...
The effects of insulin, rapamycin, and forskolin on the phosphorylation of sites was assessed by measuring the 32P contents of the appropriate peak fractions. Insulin increased the amount of 32P recovered in peptides containing Thr36, Thr45, Ser64, and Thr69 (Fig. 2). Rapamycin and forskolin had relatively small effects on the phosphorylation of PHAS-I in the absence of insulin; however, the two agents inhibited the effect of insulin on increasing the phosphorylation of the four sites. Thus, increasing cAMP with forskolin was associated with a change in the pattern of PHAS-I phosphorylation that was similar to that produced by inhibiting mTOR with rapamycin. The phosphorylation states of all four sites were also decreased by wortmannin, consistent with the effect of the PI 3-kinase inhibitor to block activation of mTOR (18).

**Inhibitory Effect of Increasing cAMP on mTOR Activation**—To determine whether increasing cAMP in cells inhibited mTOR, adipocytes were incubated with forskolin, CPT-cAMP, and theophylline before mTOR activity was measured in an immune complex assay with recombinant [H6]PHAS-I as substrate (Fig. 3). As previously reported (18), insulin increased the incubations by homogenizing the cells. PHAS-I was then immunoprecipitated from 32P-labeled adipocytes. Cells were incubated in medium containing 32P, for 3 h and then homogenized. For the final 45 min, duplicate samples of cells were incubated with no inhibitor (●), 25 nM rapamycin (▲), 100 nM wortmannin (■), or 50 μM forskolin (○). Insulin was added to one of each duplicate sample 15 min prior to terminating the incubations by homogenizing the cells. PHAS-I was then immunoprecipitated from extracts. A. samples of the immunoprecipitated protein were digested with lysyl endopeptidase before the resulting 32P-phosphopeptides were applied to a reverse phase column and eluted with an increasing gradient (dotted line) of acetonitrile. The results represent the amount of 32P recovered in fractions (1 ml), which were collected each min. LE-P1, LE-P2, and CT-P3 denote peaks of 32P-labeled peptides. B, LE-P3 fractions were pooled and incubated with chymotrypsin. The resulting 32P-phosphopeptides were isolated by reverse phase-HPLC. The results represent the amount of 32P recovered in each fraction. CT-P1 and CT-P2 denote the two peaks of 32P-labeled peptides that were resolved. C, amino acid sequences of rat and 3T3-L1 adipocyte PHAS-I proteins deduced from the nucleotide sequences of cloned cDNA cloned are shown. The phosphorylated residues and the peptides previously identified in LE-P1, LE-P2, LE-P3, CT-P1, and CT-P2 derived from PHAS-I isolated from 32P-labeled adipocytes are indicated. Residues in 3T3-L1 adipocyte PHAS-I that differ from those in the rat protein are in bold type.

**FIG. 1.** Peptide mapping of PHAS-I immunoprecipitated from 32P-labeled 3T3-L1 adipocytes. Cells were incubated in medium containing 32P, for 3 h and then homogenized. For the final 45 min, duplicate samples of cells were incubated with no inhibitor (●), 25 nM rapamycin (▲), 100 nM wortmannin (■), or 50 μM forskolin (○). Insulin was added to one of each duplicate sample 15 min prior to terminating the incubations by homogenizing the cells. PHAS-I was then immunoprecipitated from extracts. A. samples of the immunoprecipitated protein were digested with lysyl endopeptidase before the resulting 32P-phosphopeptides were applied to a reverse phase column and eluted with an increasing gradient (dotted line) of acetonitrile. The results represent the amount of 32P recovered in fractions (1 ml), which were collected each min. LE-P1, LE-P2, and CT-P3 denote peaks of 32P-labeled peptides. B, LE-P3 fractions were pooled and incubated with chymotrypsin. The resulting 32P-phosphopeptides were isolated by reverse phase-HPLC. The results represent the amount of 32P recovered in each fraction. CT-P1 and CT-P2 denote the two peaks of 32P-labeled peptides that were resolved. C, amino acid sequences of rat and 3T3-L1 adipocyte PHAS-I proteins deduced from the nucleotide sequences of cloned cDNA cloned are shown. The phosphorylated residues and the peptides previously identified in LE-P1, LE-P2, LE-P3, CT-P1, and CT-P2 derived from PHAS-I isolated from 32P-labeled adipocytes are indicated. Residues in 3T3-L1 adipocyte PHAS-I that differ from those in the rat protein are in bold type.

**FIG. 2.** Effects of insulin, rapamycin, and forskolin on the phosphorylation of sites in PHAS-I. 3T3-L1 adipocytes were incubated with 32P, and then incubated without additions or with rapamycin, forskolin, and insulin before PHAS-I was immunoprecipitated and 32P-phosphopeptides were isolated as described in the legend to Fig. 1. The amounts of 32P in HPLC fractions containing the peptides were determined by using a liquid scintillation spectrometer. Phosphorylation sites represented by the 32P-phosphopeptides recovered in HPLC fractions after proteolytic digestion of [32P]PHAS-I were as follows: Thr36 (CT-P2), Thr45 (CT-P1), Ser64 (LE-P2), and Thr69 (LE-P1). The results are expressed as percentages of the respective 32P contents of the sites from cells incubated without insulin or inhibitors. Mean values ± S.E. from four experiments are presented.

**Inhibitory Effect of Increasing cAMP on mTOR Activation**—To determine whether increasing cAMP in cells inhibited mTOR, adipocytes were incubated with forskolin, CPT-cAMP, and theophylline before mTOR activity was measured in an immune complex assay with recombinant [H6]PHAS-I as substrate (Fig. 3). As previously reported (18), insulin increased

**FIG. 3.**
the \([H^6]\) PHAS-I kinase activity of mTOR by approximately 2.5-fold. Forskolin and CPT-cAMP were without effect on mTOR activity when cells were incubated in the absence of insulin, but both agents markedly inhibited the activation of mTOR by insulin. Like forskolin and CPT-cAMP, theophylline prevented the activation of mTOR by insulin; however, theophylline also decreased basal mTOR activity, suggesting that the mechanism of its inhibitory effect differed from that of the other two agents. Further evidence for different mechanisms of action was obtained in a control experiment in which agents were included in the reaction mixture used to measure mTOR activity. Neither forskolin nor CPT-cAMP affected the PHAS-I kinase activity of mTOR immunoprecipitated from extracts of 3T3-L1 adipocytes or rat brain (16) by using mTAb1 and protein A-agarose beads. Immune complexes were incubated in mTOR Reaction Mix without additions or with 25 nM rapamycin, 0.5 mM phylline on the activation of mTOR by insulin. 3T3-L1 adipocytes 

Values are mean ± S.E. of five experiments. 

The results support the hypothesis that the agents block the activation of mTOR by preventing the phosphorylation of the protein. 

We next determined whether increasing cAMP affected the activity of PKB (Fig. 6), an upstream element in the mTOR signaling pathway (18). Phosphorylation and activation of PKB is associated with a decrease in the electrophoretic mobility of the enzyme when analyzed by SDS-PAGE. Insulin treatment of cells retarded the mobility of approximately half of the PKB, which was detected by immunoblotting (Fig. 6A). The mobility shift produced by insulin was associated with an increase of approximately 5-fold in PKB activity (Fig. 6B), assessed in an immune complex assay with histone 2B as a substrate (Fig. 6A). Neither forskolin nor CPT-cAMP decreased PKB activity when added to adipocytes incubated without insulin or when added to cells prior to insulin (Fig. 6B). Consistent with these observations, neither agent affected the electrophoretic mobility of PKB (Fig. 6A). In contrast, theophylline blocked the gel shift produced by insulin and ablated the stimulatory effect of the hormone on PKB activity.

Figure 3. Inhibitory effects of forskolin, CPT-cAMP, and theophylline on the activation of mTOR by insulin. 3T3-L1 adipocytes were incubated without additions or with 25 nM rapamycin, 0.5 mM CPT-cAMP, or 5 mM theophylline for 30 min. After addition of insulin (10 milliunits/ml) as indicated, the incubations were continued for 15 min. mTOR was immunoprecipitated with mTAb2 and then incubated in mTOR Reaction Mix for 30 min. Samples were subjected to SDS-PAGE. A, an mTAb2 immunoblot showing the immunoprecipitated mTOR and an autoradiogram showing the \(^{32}\)P-labeled \([H^6]\)PHAS-I product are shown. B, mTAb2 immunoblots were used to determine the relative amounts of mTOR immunoprecipitated. After correcting for differences in mTOR among samples, mTOR activities were determined from the amounts of \(^{32}\)P incorporated into \([H^6]\)PHAS-I. The results are expressed as percentages of the control and are mean values ± S.E. from three experiments.

Figure 4. Effects of incubating immunoprecipitated mTOR with forskolin, CPT-cAMP, and theophylline on mTOR activity.

mTOR was immunoprecipitated from extracts of 3T3-L1 adipocytes or rat brain (16) by using mTAb1 and protein A-agarose beads. Immune complexes were incubated in mTOR Reaction Mix without additions or with 50 μM forskolin, 0.5 mM CPT-cAMP, or 5 mM theophylline. Results are expressed as percentages of the respective control activities. A representative experiment is presented for 3T3-L1 mTOR. The results with rat brain mTOR are mean values ± S.E. from three experiments.
A, an immunoblot showing PKB and an autoradiogram of the $^{32}$P-measured in an immune complex assay using histone 2B as substrate. 

Legend to Fig. 3. PKB was immunoprecipitated, and PKB activity was assayed by insulin. 

3T3-L1 adipocytes were incubated as described in the legend to Fig. 3. PKB was immunoprecipitated, and PKB activity was assayed by insulin. 

**DISCUSSION**

The present results with forskolin and CPT-cAMP indicate that increasing intracellular cAMP leads to attenuation of the effect of insulin on activating mTOR. As mTOR is known to have a central role in the control of both mRNA translation and cell proliferation, an important implication is that inhibition of mTOR is involved in the potent inhibitory effects of cAMP on these processes in certain cell types. Although the control by mTOR may be complex, some of the mechanisms involved have been elucidated and provide at least a partial explanation of how cAMP-dependent inhibition of mTOR could lead to inhibition of protein synthesis and mitogenesis.

Based on studies with rapamycin, it is clear that inhibiting mTOR would lead to dephosphorylation of the translational regulators, PHAS-I and p70S6K (9, 15). Decreased phosphorylation of both proteins has been shown to occur in response to increased cAMP, at least in certain cell types (20–23). Dephosphorylation of the appropriate sites in p70S6K results in inactivation of the kinase, less phosphorylation of ribosomal protein S6, and reduced translation of those messages having the polypyrimidine tract motif (9). Dephosphorylation of PHAS-I results in increased binding of PHAS-I to eIF4E and inhibition of the translation of capped mRNA (15). Overexpressing eIF4E in cells may have a potent mitogenic effect, suggesting that eIF4E is involved in the control of cell proliferation (32, 33). Our studies do not directly address this possibility, but it is interesting to speculate that cAMP-induced inhibition of mTOR and the decrease in eIF4E function resulting from dephosphorylation of PHAS-I might contribute to the antiproliferative effects of increased cAMP. Another mechanism through which phosphorylated mTOR signaling could affect cell cycle progression involves p27Kip1, a heat-stable inhibitor of G1 cyclin-Cdk activities. Inhibiting mTOR with rapamycin results in stabilization of p27Kip1, thereby preventing the increase in cyclin-Cdk activities needed for G1 progression (34, 35). Interestingly, cAMP has also been shown to increase p27Kip1 (35), and it is an intriguing hypothesis that the actions of both rapamycin and cAMP on the inhibitor result from inhibition of mTOR. Arguing against this hypothesis is evidence that the mechanisms by which rapamycin and cAMP increase p27Kip1 are different (35). Rapamycin appears to decrease the rate at which p27Kip1 is degraded (34), whereas increased cAMP has been reported to increase the synthesis of the inhibitor (35).

The conclusion that inhibition of mTOR contributes to the effects of increased cAMP on PHAS-I and p70S6K does not imply that cAMP lacks effects on the phosphorylation of these proteins by other mechanisms. The observation that forskolin promoted a greater decrease than rapamycin in the phosphorylation of Thr45 and Thr36 would be consistent with the involvement of an mTOR-independent effect of cAMP on these two sites in PHAS-I.

The activation of p70S6K in 3T3-L1 adipocytes is attenuated by the MAP kinase kinase inhibitor, PD 098059, suggesting that one or more members of the MAP kinase family may be involved in the control of p70S6K (36). Increasing cAMP has been shown to inhibit the MAP kinase signaling pathway in several cell types (37). Interestingly, the inhibitory effect of cAMP on MAP kinase activation is not observed in all cells. Likewise, in contrast to our findings in 3T3-L1 adipocytes, neither forskolin nor cAMP derivatives decreased the activation of p70S6K in Swiss 3T3 cells (10, 38, 39). In these cells, the activation of p70S6K was still markedly inhibited by methylxanthine phosphodiesterase inhibitors, even though cAMP levels were not significantly changed. These results support the conclusion that methylxanthines act by a cAMP-independent mechanism to block activation of p70S6K. Based on the fact that methylxanthines and agents that increase cAMP (10, 38, 39). This is unlikely to be the case, as experiments in several cell types, including 3T3-L1 adipocytes, have shown that cAMP derivatives and agents that increase cAMP markedly inhibit activation of p70S6K in the absence of methylxanthines (20, 21, 23). The most straightforward interpretation of these results is that the effects of methylxanthines and increased cAMP are mediated by at least two different mechanisms. Methylxanthines could inhibit activation of mTOR by blocking activation of the upstream effector, PKB (Fig. 6). However, based on the finding that theophylline markedly inhibited mTOR activity in vitro (Fig. 4), we propose that methylxanthines directly inhibit mTOR. Because this direct mechanism would presumably operate in all cells expressing mTOR, it could account for the inhibitory effects of methylxanthines on the phosphorylation of p70S6K and PHAS-I in cells lacking the response to cAMP. As discussed below, increasing cAMP presumably acts by activating PKA, which phosphorylates an upstream regulator of mTOR phosphorylation. The indirect mechanism could explain why the response to cAMP is not observed in all cell types, since factors such as the level of expression of regulatory elements or the presence of opposing pathways could determine whether mTOR activity was decreased.

Studies from several laboratories have implicated activation of PKB in the effects of insulin on PHAS-I (19, 40, 41), and we have recently obtained evidence implicating PKB in the activation of mTOR by insulin (18). Therefore, we considered the possibility that increasing cAMP might inhibit activation of mTOR by preventing the activation of PKB. This hypothesis can be eliminated since neither forskolin nor CPT-cAMP affected the activation of PKB in response to insulin (Fig. 6) under conditions in which the agents markedly attenuated the activation of mTOR by the hormone (Fig. 3). The failure to
affect PKB activation also indicates that inhibition of upstream signaling from the insulin receptor cannot explain the actions of cAMP. mTOR contains consensus sites for phosphorylation by PKA (42), and we have attempted to test the hypothesis that mTOR is inactivated by PKA. However, we were not able to phosphorylate mTOR in vitro with purified catalytic subunit of PKA or to affect mTOR kinase activity by incubating mTOR immune complexes with the catalytic subunit. These negative findings do not eliminate the possibility that PKA can phosphorylate mTOR under the appropriate conditions. However, the finding that increased cAMP opposes the effect of insulin on mTOR phosphorylation, as assessed by mTAb1 binding (18), provides further support for the interpretation that the effects of cAMP are not mediated by direct phosphorylation of mTOR by PKA.

The recent finding that activation of PKB by tamoxifen in MER-Akt cells resulted in activation of mTOR and the loss of mTAb1 binding indicates that activation of PKB is sufficient for the activation of mTOR, at least in some cell types (18). As discussed previously (18), the mTAb1 epitope contains an almost ideal consensus site for phosphorylation by PKB, but we have not been able to demonstrate direct phosphorylation of this site by PKB. Thus, although there is a strong argument for placing PKB upstream of mTOR in the signaling pathway, PKB may not directly phosphorylate mTOR. In order to understand the action of cAMP, it will be necessary to identify the protein kinase(s) responsible for the phosphorylation and activation of mTOR. As a working hypothesis, we suggest that there is an intervening kinase, designated TORK, between PKB and mTOR. A model in which activation of PKB or PKA resulted in activation or inhibition of TORK would be consistent with the available results pertaining to the regulation of mTOR by insulin and cAMP.

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