

Signaling Elements Involved in the Metabolic Regulation of mTOR by Nutrients, Incretins, and Growth Factors in Islets

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Mammalian target of rapamycin (mTOR) is a protein kinase that integrates signals from mitogens and the nutrients, glucose and amino acids, to regulate cellular growth and proliferation. Previous findings demonstrated that glucose robustly activates mTOR in an amino acid-dependent manner in rodent and human islets. Furthermore, activation of mTOR by glucose significantly increases rodent islet DNA synthesis that is abolished by rapamycin. Glucagon-like peptide-1 (GLP-1) agonists, through the production of cAMP, have been shown to enhance glucose-dependent proinsulin biosynthesis and secretion and to stimulate cellular growth and proliferation. The objective of this study was to determine if the glucose-dependent and cAMP-mediated mechanism by which GLP-1 agonists enhance β -cell growth and proliferation is mediated, in part, through mTOR. Our studies demonstrated that forskolin-generated cAMP resulted in activation of mTOR at basal glucose concentrations as assessed by phosphorylation of S6K1, a downstream effector of mTOR. Conversely, an adenylyl cyclase inhibitor partially blocked glucose-induced S6K1 phosphorylation. Furthermore, the GLP-1 receptor agonist, Exenatide, dose-dependently enhanced phosphorylation of S6K1 at an intermediate glucose concentration (8 mmol/l) in a rapamycin-sensitive manner. To determine the mechanism responsible for this potentiation of mTOR, the effects of intra- and extracellular Ca^{2+} were examined. Glyburide, an inhibitor of ATP-sensitive K^+ channels (K_{ATP} channels), provided partial activation of mTOR at basal glucose concentrations due to the influx of extracellular Ca^{2+} , and diazoxide, an activator of K_{ATP} channels, resulted in partial inhibition of S6K1 phosphorylation by 20 mmol/l glucose. Furthermore, Exenatide or forskolin reversed the inhibition by diazoxide, probably through mobiliza-

tion of intracellular Ca^{2+} stores by cAMP. BAPTA, a chelator of intracellular Ca^{2+} , resulted in inhibition of glucose-stimulated S6K1 phosphorylation due to a reduction in cytosolic Ca^{2+} concentrations. Selective blockade of glucose-stimulated Ca^{2+} influx unmasked a protein kinase A (PKA)-sensitive component involved in the mobilization of intracellular Ca^{2+} stores, as revealed with the PKA inhibitor H-89. Overall, these studies support our hypothesis that incretin-derived cAMP participates in the metabolic activation of mTOR by mobilizing intracellular Ca^{2+} stores that upregulate mitochondrial dehydrogenases and result in enhanced ATP production. ATP can then modulate K_{ATP} channels, serve as a substrate for adenylyl cyclase, and possibly directly regulate mTOR activation. *Diabetes* 53 (Suppl. 3):S225–S232, 2004

Mammalian target of rapamycin (mTOR) is a serine and threonine protein kinase that regulates protein translation through a rapamycin-sensitive pathway involving the regulatory proteins 70-kDa ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor 4E-binding protein-1 (4EBP1) also designated as PHAS-I (Fig. 1) (rev. in 1). Rapamycin is a specific inhibitor of mTOR function and thus a valuable tool for the characterization of this pathway. mTOR is unique in that it integrates signals from mitogens and the nutrients—glucose and amino acids—to regulate cellular growth. This is accomplished, in part, by the ability of mTOR to stimulate protein synthesis by increasing mRNA translation initiation and the capacity of the ribosomal protein machinery (2,3). The regulation of protein synthesis is central to cell replication because reentry of cells into the cell cycle in response to nutrients and growth factors is accompanied by doubling of protein synthesis before the onset of DNA synthesis (4–6) and cell division (6,7). Although mTOR is ubiquitous from yeast to mammals, there may be specific signaling aspects unique to β -cells. Our previous findings have shown that glucose in the elevated physiological range robustly activates mTOR in an amino acid-dependent manner in both rodent and human islets (8–10). mTOR is also activated by the insulin receptor signaling cascade. However, mTOR can be activated by nutrients independent of insulin and other growth factors. Our previous studies indicate that optimization of mitochondrial function is required for the ability of nutrients to activate mTOR, although the precise mechanism that couples mTOR activity to mitochondrial function is unknown (11).

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AMPK, AMP-dependent protein kinase; CMRL, CMRL-1066 medium; cCMRL, CMRL plus 5.6 mmol/l glucose, supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin; eIF-4E, eukaryotic initiation factor 4E; GLP-1, glucagon-like peptide-1; IRS, insulin receptor substrate; K_{ATP} channel, ATP-sensitive K^+ channel; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent kinase; PI3K, phosphoinositide-3 kinase; PKA, protein kinase A; PKB, protein kinase B.

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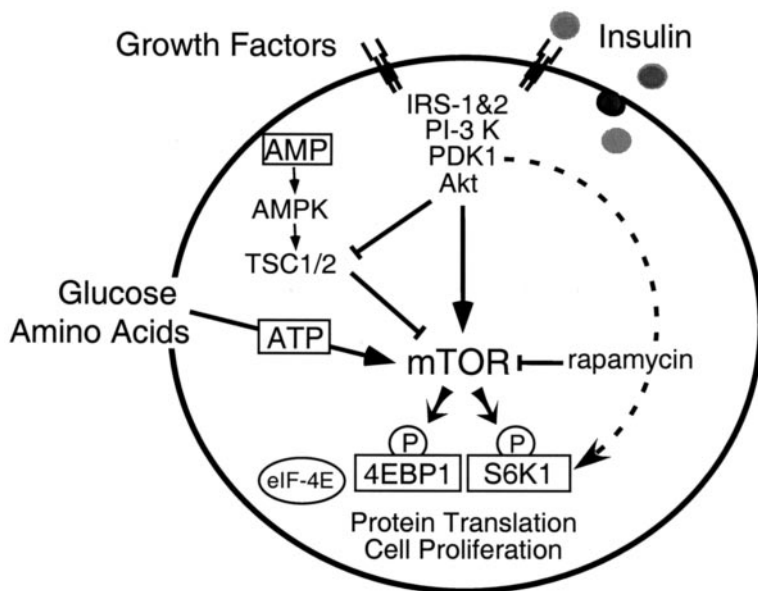


FIG. 1. Proposed model of metabolic and autocrine regulation of the mTOR pathway by β -cells. See text for description. TSC1 (also known as hamartin) and TSC2 (also known as tuberlin) are protein products of mutated tuberous sclerosis genes *TSC1* and *TSC2*.

The significance of the mTOR pathway for growth and survival of β -cells is emphasized in Fig. 2. In this experimental design, rapamycin dose-dependently inhibited ^3H -thymidine incorporation, as an indicator of DNA synthesis, following a 4-day exposure of isolated rat islets to elevated glucose. Rapamycin-induced inhibition of ^3H -thymidine incorporation was also observed at basal glucose concentrations, suggesting that basal glucose metabolism by islets also exerts a regulatory role on mTOR activation. This inhibition of glucose-induced DNA synthesis by rapamycin is consistent with the unique role of mTOR to regulate growth and proliferation by sensing nutrient availability. The importance of appropriate regulation of mTOR is illustrated in Fig. 2B. mTOR remained fully activated in isolated rat islets during continuous exposure to an elevated glucose concentration for 4 or 6 days, and rapamycin completely inhibited this activation. Continuous activation of mTOR during conditions of hyperglycemia or inhibition due to immunosuppression with rapamycin could have long-term implications for changing gene expression patterns. In addition, our previous findings show that rapamycin inhibits glucose-induced protein synthesis in rodent islets (9). Moreover, S6K1, a target of mTOR, is proposed to control cell size by increasing mRNA translation. This concept is supported by studies with S6K1-deficient mice, which display hypoinsulinemia, glucose intolerance, and diminished β -cell size (12).

GLP-1 exerts a variety of effects on β -cells, although the mechanisms of action are not completely understood (rev. in 13,14). GLP-1 receptor agonists, through activation of G protein-coupled receptors, enhance glucose-dependent proinsulin biosynthesis, insulin secretion, stimulate cellular growth, and proliferation, and reduce apoptosis in a variety of in vivo models by a glucose-dependent or -associated mechanism (15–18). GLP-1 action on the $\text{Gs}\alpha$ subunit activates adenylyl cyclases to increase cAMP levels, while the $\text{Gs}\beta\gamma$ dimer activates phosphoinositide-3 kinase (PI3K) and, subsequently, the transcription factor PDX-1, and possibly mitogen-activated protein kinase (MAPK) (19). Exenatide (synthetic Exendin-4), an agonist of the GLP-1 receptor that is resistant to proteolytic

enzymes, mimics the positive effects of GLP-1 on β -cells in rodents (20).

The similarity between the glucose dependency of GLP-like hormones and mTOR activation, in particular for enhanced growth and proliferation, is striking. Based on this similarity, the focus of this study was to determine if the glucose dependency and/or associated mechanisms by which GLP-1 receptor agonists enhance β -cell growth and proliferation are mediated, in part, through activation of mTOR. A major thrust of this experimental approach was to determine if the ability of GLP-1 receptor agonists to increase cAMP production results in synergistic effects with glucose on β -cell growth and proliferation through mTOR signaling. Overall, our purpose was to achieve a more thorough knowledge of the mTOR-signaling pathway in order to determine potential sites for therapeutic intervention to promote β -cell survival, growth, and proliferation.

ROLE OF cAMP

Our initial approach was to assess the effects of forskolin-generated cAMP on S6K1 phosphorylation at basal glucose concentrations. In this experimental design, gel shift assays were used to detect mTOR-mediated phosphorylation of S6K1. As shown in Fig. 3A, incubation of isolated rat islets for 60 min in CMRL tissue culture medium in the presence of an elevated glucose concentration (20 mmol/l) resulted in enhanced phosphorylation of S6K1 (lane 2) as visualized by the appearance of a more slowly migrating upper band in comparison to 3 mmol/l glucose (lane 1). As shown in lanes 3–5, forskolin dose-dependently increased phosphorylation of S6K1 with optimal effects observed at 1 and 10 $\mu\text{mol/l}$ forskolin. The ability of cAMP to enhance S6K1 phosphorylation was independent of insulin secretion and blocked by rapamycin (data not shown). Studies were also performed with MDL-12330A, an adenylyl cyclase inhibitor (21), to determine if the ability of 20 mmol/l glucose to robustly activate mTOR involved the production of cAMP. As shown in Fig. 3B, MDL-12330A dose-dependently reduced glucose-stimulated phos-

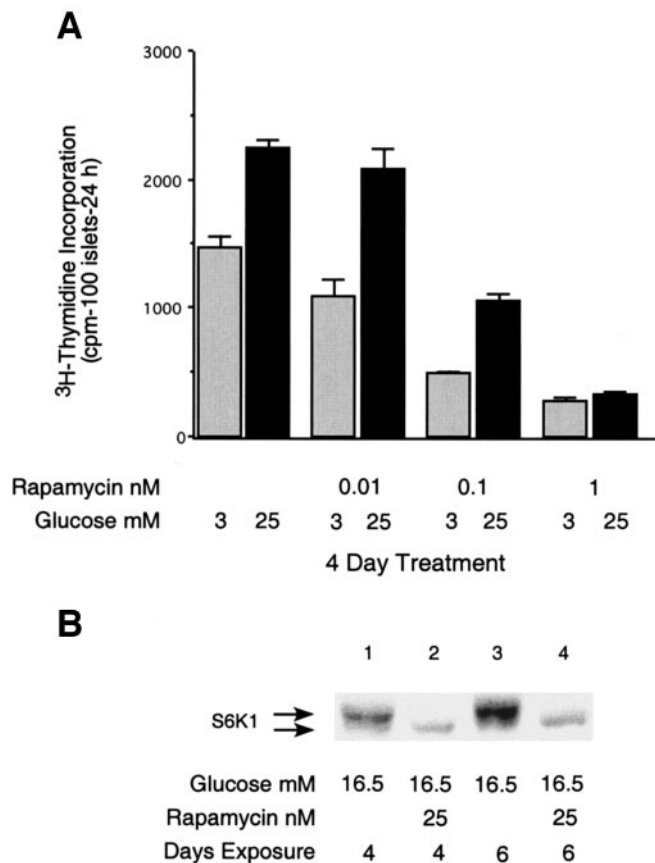


FIG. 2. Glucose enhances DNA synthesis and S6K1 phosphorylation in a rapamycin-sensitive manner. **A:** Islets were isolated from Sprague-Dawley rats (~200 g) and counted ($n = 100$) into Petri dishes (35×10 mm). Islets were cultured for 4 days in 1 ml of CMRL, 3 or 25 mmol/l glucose, 10% FCS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin \pm rapamycin as indicated at 37°C, under 95% air/5% CO₂. The medium was not changed during this time. During the final 24 h of incubation, ³H-thymidine was added to each dish. ³H-thymidine incorporation was determined by trichloroacetic acid extraction and scintillation counting. Data are representative of two experiments at 0.01–1.0 nmol/l rapamycin with triplicate dishes in each experiment. Additional studies were performed at 10 nmol/l rapamycin ($n = 3$) and at 25 nmol/l rapamycin ($n = 2$) under similar conditions (data not shown). In each experiment, three replicate dishes were used for each condition. **B:** Rat islets were cultured overnight in cMRL medium, 10 mmol/l glucose. Islets ($n = 200$) were then incubated in 3 ml of cMRL, 16.5 mmol/l glucose \pm 25 nmol/l rapamycin, for 4 or 6 days in 60×15 mm Petri dishes at 37°C, under 95% air/5% CO₂. The media was not changed during this time. Following incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Blot is representative of three experiments.

phosphorylation of S6K1 with a marked inhibition observed at 15 μ mol/l (lane 5 vs. lane 2). Under similar conditions, MDL-12330A inhibited forskolin-induced cAMP production by ~80% (data not shown). This adenylyl cyclase inhibitor had no effect on glucose-stimulated insulin secretion, suggesting that it was not exerting nonspecific effects at these concentrations. These studies also demonstrated that although cAMP increases insulin secretion in a glucose-dependent manner, and thus provides a synergistic, autocrine effect on S6K1 phosphorylation through the insulin-signaling pathway, it also exerts an effect on mTOR independent of insulin secretion. Additionally, cAMP analogs at basal glucose concentrations resulted in phosphorylation of S6K1 comparable to 20 mmol/l glucose (data not shown).

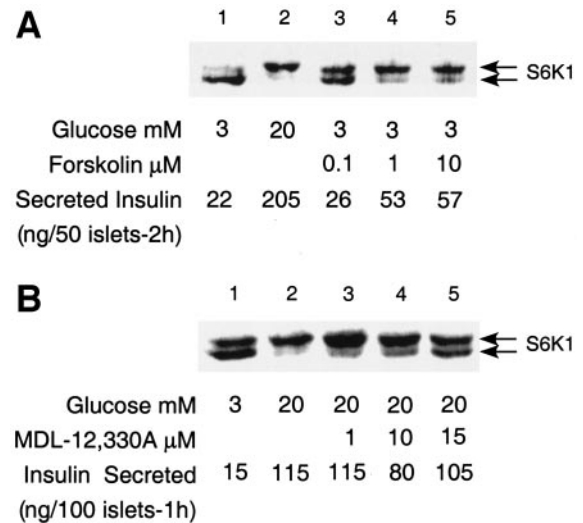


FIG. 3. The effects of cAMP on phosphorylation of S6K1 under basal and elevated glucose concentrations. **A:** Rat islets were cultured overnight in cMRL medium, 5.6 mmol/l glucose. Islets (50/500 μ l in untreated 12-well BD Falcon plates) were washed and incubated for 30 min in CMRL + 3 mmol/l glucose + 0.1% BSA at 37°C, under 95% air/5% CO₂. Medium was changed and islets incubated for 60 min in CMRL, 3 or 20 mmol/l glucose \pm forskolin, as indicated. Following incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Blot is representative of five experiments. **B:** Rat islets were cultured overnight in cMRL medium, 5.6 mmol/l glucose. Islets (100/500 μ l in untreated 12-well BD Falcon plates) were washed and incubated for 30 min in CMRL + 3 mmol/l glucose + 0.1% BSA \pm MDL-12330A HCl at 37°C, under 95% air/5% CO₂. Medium was changed and islets incubated for 60 min in CMRL, 3 or 20 mmol/l glucose \pm MDL-12330A HCl, as indicated. Following incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Blot is representative of four experiments.

EXENATIDE ENHANCES GLUCOSE-INDUCED PHOSPHORYLATION OF S6K1 IN ISOLATED RAT ISLETS

To further characterize the effects of Exenatide on the ability of glucose to stimulate S6K1 phosphorylation via mTOR activation, a dose-response of Exenatide was performed at an intermediate concentration of glucose. As shown in Fig. 4, 8 mmol/l glucose resulted in a partial activation of S6K1 (lane 2 vs. lane 3) and Exenatide at 1, 10, and 100 nmol/l (lanes 4–6) dose-dependently stimulated S6K1 phosphorylation at 8 mmol/l glucose, comparable to that attained at 20 mmol/l glucose. Exenatide (10 or 100 nmol/l) was ineffective in stimulating S6K1 phosphorylation at 3 mmol/l glucose and failed to further enhance S6K1 phosphorylation at 15 or 20 mmol/l glucose, and rapamycin (25 nmol/l) inhibited completely the enhanced phosphorylation of S6K1 at all glucose concentrations (data not shown). These studies demonstrate that the glucose-dependency of Exenatide to stimulate S6K1 phosphorylation is, in fact, mediated through activation of mTOR. Overall, these findings suggest that the ability of GLP-1 agonists to increase cAMP production results in synergistic effects with glucose that may contribute to β -cell growth and survival through mTOR.

ROLE OF Ca²⁺

GLP-1 increases cAMP production, induces Ca²⁺ influx through L-type voltage-gated Ca²⁺ channels, and causes Ca²⁺-induced Ca²⁺ release (CICR) from intracellular stores

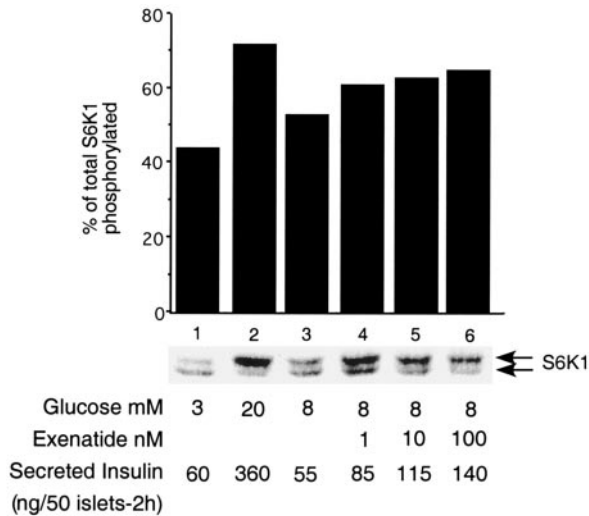


FIG. 4. Exenatide enhances glucose-induced phosphorylation of S6K1. Rat islets were cultured overnight in cCMRL medium, 5.6 mmol/l glucose. Islets (50/500 μ l in untreated 12-well BD Falcon plates) were washed and incubated for 120 min in CMRL + 0.1% BSA, 3, 8, or 20 mmol/l glucose \pm Exenatide, as indicated, at 37°C, under 95% air/5% CO₂. After incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Bars indicate percent of total S6K1 that is phosphorylated for this Western blot only. Blot is representative of three experiments.

(22,23). To assess the effect of enhanced Ca²⁺ influx on mTOR activation, isolated rat islets were exposed to glyburide (1 μ mol/l) at a basal glucose concentration (3 mmol/l). As shown in Fig. 5, inhibition of K_{ATP} channels by glyburide resulted in a partial stimulation of S6K1 phosphorylation (*lane 4 vs. lane 1*) as indicated by the appearance of a more slowly migrating upper band. This increased phosphorylation of S6K1 was completely blocked by rapamycin (*lane 5*). Glyburide also increased insulin secretion at basal glucose concentrations. Although insulin secretion in response to 1 μ mol/l glyburide was similar to 20 mmol/l glucose, the level of phosphorylation of S6K1 was only partial. Thus, elevated glucose provides an additional signal to mTOR separate from the insulin secretory signal. Overall, these findings suggest that increases in both

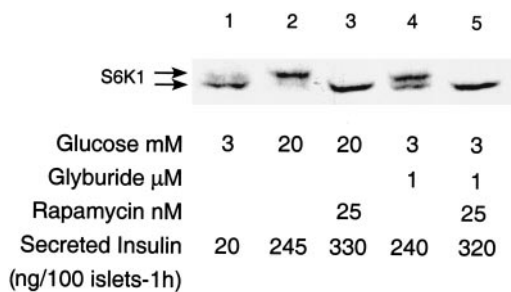


FIG. 5. Glyburide-induced closure of K_{ATP} channels causes partial phosphorylation of S6K1. Rat islets were cultured overnight in cCMRL medium, 5.6 mmol/l glucose. Islets (100/500 μ l in untreated 12-well BD Falcon plates) were washed and incubated for 30 min in CMRL + 0.1% BSA, 3 mmol/l glucose \pm rapamycin, as indicated, at 37°C, under 95% air/5% CO₂. Medium was changed and islets incubated for 60 min in CMRL + 0.1% BSA, 3 or 20 mmol/l glucose \pm glyburide \pm rapamycin as indicated. After incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Blot is representative of three experiments.

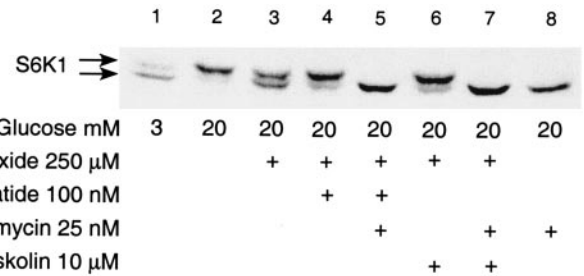


FIG. 6. Exenatide and forskolin restore phosphorylation of S6K1 inhibited by diazoxide. Rat islets were cultured overnight in cCMRL medium, 5.6 mmol/l glucose. Islets (100/500 μ l in untreated 12-well BD Falcon plates) were washed and incubated for 30 min in CMRL + 0.1% BSA, 3 mmol/l glucose \pm diazoxide \pm rapamycin, as indicated, at 37°C, under 95% air/5% CO₂. Medium was changed and islets incubated for 60 min in CMRL + 0.1% BSA, 3 or 20 mmol/l glucose \pm diazoxide \pm Exenatide \pm forskolin \pm rapamycin, as indicated. After incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Blot is representative of five experiments.

intracellular cAMP and Ca²⁺ may be involved in the metabolic activation of mTOR.

EFFECT OF DIAZOXIDE ON cAMP-MEDIATED PHOSPHORYLATION OF S6K1

Our next approach was to examine the effects of preventing the opening of L-type Ca²⁺ channels with diazoxide, an activator of K_{ATP} channels, on glucose-stimulated S6K1 phosphorylation. Because glyburide, an inhibitor of K_{ATP} channels, resulted in a partial activation of mTOR as assessed by increased phosphorylation of S6K1, the expected result due to diazoxide exposure was an attenuation of enhanced S6K1 phosphorylation by glucose. An advantage of this model of hyperpolarization is that diazoxide prevents glucose-stimulated insulin secretion due to activation of K_{ATP} channels but does not alter glucose-stimulated metabolism (24). Thus, the ability of an elevated concentration of glucose to upregulate the mitochondria remains intact. As shown in Fig. 6, diazoxide treatment resulted in a partial inhibition of S6K1 phosphorylation by 20 mmol/l glucose (*lane 3 vs. lane 2*). Furthermore, cAMP derived from Exenatide (100 nmol/l) or forskolin (10 μ mol/l), as shown in *lanes 4 and 6*, completely reversed this inhibition in a rapamycin-sensitive manner (*lanes 5 and 7*). Glucose-stimulated insulin secretion was blocked by diazoxide even when S6K1 phosphorylation was restored with Exenatide, and forskolin partially restored glucose-stimulated insulin secretion to ~50% of control values obtained with 20 mmol/l glucose (data not shown). Because diazoxide prevents Ca²⁺ influx due to activation of K_{ATP} channels, the ability of cAMP to reverse inhibition of S6K1 phosphorylation is believed to be due primarily to the mobilization of intracellular Ca²⁺ stores.

cAMP INDUCES S6K1 PHOSPHORYLATION BY THE RELEASE OF INTRACELLULAR Ca²⁺

To further support a role for cAMP-induced mobilization of intracellular Ca²⁺ as a mediator of mTOR activation, we examined the effect of chelating intracellular Ca²⁺ with BAPTA. In this experimental design, diazoxide was used to prevent the influx of extracellular Ca²⁺ in the presence of an elevated glucose concentration. As shown in Fig. 7A, diazoxide partially inhibited the ability of 20 mmol/l glu-

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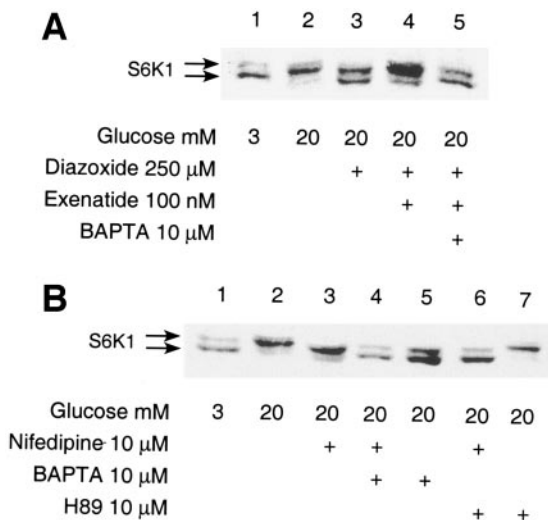


FIG. 7. Reducing cytosolic calcium inhibits phosphorylation of S6K1. A: Rat islets were cultured overnight in cMRL medium, 5.6 mmol/l glucose. Islets (100/1 ml in untreated 35×10 mm BD Falcon Petri dishes) were washed and incubated for 30 min in CMRL + 0.1% BSA, 3 mmol/l glucose \pm diazoxide \pm Exenatide \pm BAPTA, as indicated, at 37°C, under 95% air/5% CO₂. Medium was changed and islets incubated for 60 min in CMRL + 0.1% BSA, 3 or 20 mmol/l glucose \pm diazoxide \pm Exenatide \pm BAPTA, as indicated. After incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Blot is representative of four experiments. **B:** Rat islets were cultured overnight in cMRL medium, 5.6 mmol/l glucose. Islets (100/500 μ l in untreated 12-well BD Falcon plates) were washed and incubated for 30 min in CMRL + 0.1% BSA, 3 mmol/l glucose \pm nifedipine \pm H89 \pm BAPTA, as indicated, at 37°C, under 95% air/5% CO₂. Medium was changed and islets incubated for 60 min in CMRL + 0.1% BSA, 3 or 20 mmol/l glucose \pm nifedipine \pm H89 \pm BAPTA, as indicated. After incubation, islets were processed for SDS-PAGE electrophoresis and Western blotting for S6K1. Culture medium was used to measure insulin secretion by radioimmunoassay. Blot is representative of three experiments.

cose to stimulate S6K1 phosphorylation (*lanes 2 and 3*). Exenatide reversed this inhibition (*lane 4*), and the presence of BAPTA (10 μ mol/l) in combination with Exenatide resulted in nearly complete inhibition of S6K1 phosphorylation (*lane 5*).

Studies were also performed with nifedipine, an inhibitor of L-type voltage-gated Ca²⁺ channels. In Fig. 7B, nifedipine (10 μ mol/l) alone failed to inhibit glucose-stimulated S6K1 phosphorylation although glucose-stimulated insulin secretion was inhibited to basal levels (data not shown). These studies suggest that although nifedipine blocks the influx of extracellular Ca²⁺ sufficiently to inhibit glucose-stimulated insulin secretion, it failed to reduce cytosolic Ca²⁺ concentrations to a level necessary to block glucose-stimulated phosphorylation of S6K1. Furthermore, BAPTA alone or in combination with nifedipine resulted in the same magnitude of inhibition, suggesting that BAPTA sufficiently reduced cytosolic Ca²⁺ concentrations to inhibit glucose-stimulated S6K1 phosphorylation. The ability of nifedipine to block the influx of extracellular Ca²⁺ also revealed a protein kinase A (PKA)-sensitive component involved in the mobilization of intracellular Ca²⁺ stores, as unmasked by the PKA inhibitor H-89 (Fig. 7B, *lane 3* vs. *lane 6*). Overall, these results support our hypothesis that the mobilization of Ca²⁺ from intracellular stores by Exenatide or forskolin is essential for the subsequent activation of mTOR by glucose under conditions when Ca²⁺ influx is inhibited.

METABOLIC REGULATION OF mTOR SIGNALING

The proposed mechanism for the metabolic regulation of mTOR is depicted schematically in Fig. 8. In this model, mTOR is activated optimally by integrating components of several pathways. First, the metabolism of glucose and amino acids, in particular the branched chain amino acid leucine, provides TCA intermediates that are used by the mitochondria to generate ATP. ATP also serves as a substrate for adenylyl cyclase. Second, ATP in combination with ADP inhibits K_{ATP} channels that result in membrane depolarization and influx of extracellular Ca²⁺. This increase in cytosolic Ca²⁺ facilitates CICR from intracellular stores as follows. Extracellular Ca²⁺ influx stimulates adenylyl cyclase, which uses ATP generated by glycolysis and mitochondrial metabolism to generate cAMP, which subsequently mobilizes intracellular Ca²⁺ stores by a

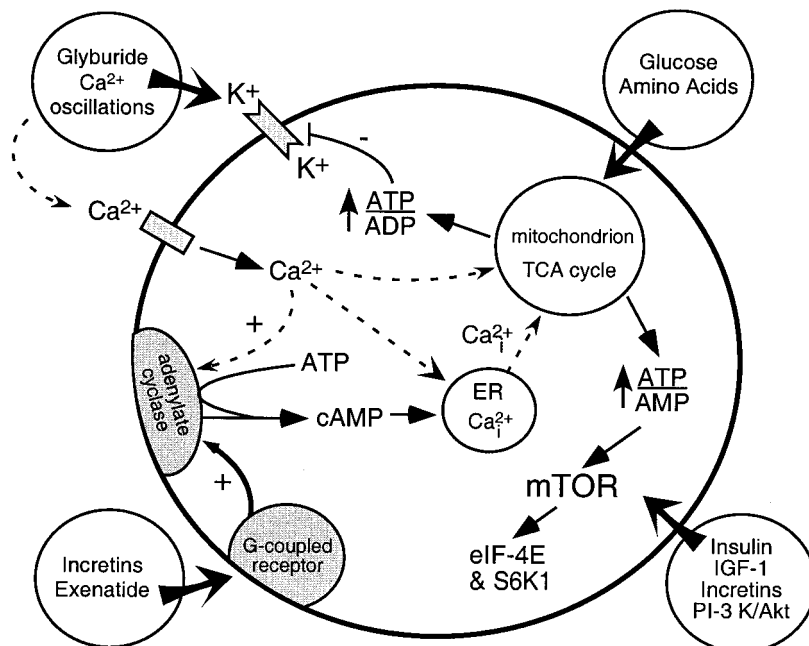


FIG. 8. Potential therapeutic intervention sites for optimal activation of islet mTOR. See text for description. Ca²⁺, intracellular Ca²⁺ concentration.

PKA-dependent mechanism and, possibly, a PKA-independent mechanism. Overall, this enhanced increase in cytosolic Ca^{2+} derived from the extracellular media and intracellular stores optimally energizes the mitochondria by activating mitochondrial dehydrogenases.

Consistent with this proposed mechanism, glyburide provided partial activation of mTOR under basal glucose concentrations because of the lack of TCA intermediate provision to the mitochondria at 3 mmol/l glucose. Support for modulation of mTOR by cAMP is also provided by the ability of forskolin to promote S6K1 phosphorylation at basal glucose concentrations. Additionally, an adenylyl cyclase inhibitor partially blocked glucose-induced S6K1 phosphorylation. Further support for modulation of mTOR by cAMP is provided by the diazoxide-hyperpolarization model in which Ca^{2+} influx is inhibited although the metabolism of glucose is unaltered (Fig. 6). Inhibition of extracellular Ca^{2+} influx results in a lower generation of cAMP that does not allow for CICR and the upregulation of mitochondrial dehydrogenases for maximal ATP production. Thus, elevated glucose resulted in only partial activation of mTOR. However, the production of cAMP, generated by Exenatide or forskolin, mobilized intracellular Ca^{2+} stores under conditions of reduced extracellular Ca^{2+} influx and produced full activation of mTOR in a BAPTA-sensitive manner. This may be explained by the increase in mitochondria-derived ATP that opposes the effects of diazoxide on K_{ATP} channels. Exenatide may also be acting through the $\text{Gs}\beta\gamma$ portion of the GLP-1 receptor to stimulate the PI3K and Akt pathway. There is also the possibility that cAMP may have a direct effect on K_{ATP} channels.

POTENTIAL MEDIATORS OF mTOR SIGNALING

Although the mitochondrial mediators of mTOR activation are not well characterized, enhanced ATP production is a prime candidate. Our previous studies have indicated that agents that interfere with oxidative phosphorylation result in the inability of glucose to activate mTOR (11). Other studies have demonstrated that interactions of mTOR with the mitochondria in some manner permit mTOR to respond to changes in mitochondrial function, including conditions of osmotic stress and hypoxia (25,26). Recently, mTOR was reported to serve as a physiological homeostatic ATP sensor (27). However, arguments against this hypothesis include the relatively high cellular content of ATP and its maintenance over a narrow concentration range. Of particular relevance to this hypothesis, Tsuboi et al. (28) demonstrated that GLP-1 increases mitochondrial concentrations of both Ca^{2+} and ATP in the β -cell line MIN6 under basal glucose concentrations. They propose that GLP-1 evokes the release of intracellular Ca^{2+} stores in a cAMP-dependent manner that leads to increase in mitochondria-localized Ca^{2+} and to a stable 1.5- to 2.0-fold increase in ATP synthesis. Their results suggest that β -cells specialize in coupling changes in electrical activity to glucose metabolism to regulate insulin secretion and may depend on mitochondria-derived ATP, compartmentalized in a manner unique from other cell types. Although Tsuboi et al. (28) focused on the mechanism of GLP-1 potentiation of glucose-induced insulin secretion, their findings are also pertinent to the metabolic regulation of mTOR. Another potential candidate as mediator of mTOR

activation is AMP, which is argued to serve as a more sensitive indicator of the β -cell energy status than ATP. Recent studies have proposed, as described in Fig. 1, that AMP-dependent protein kinase (AMPK) suppresses mTOR through the TSC1/2 complex. There may also be a role for Rheb/GTP signaling to mTOR (rev. in 1).

CHARACTERISTICS OF GLUCOSE-STIMULATED mTOR SIGNALING AND INSULIN SECRETION

Although glucose-stimulated insulin secretion and mTOR activation utilize similar signaling pathways, the following significant differences exist: 1) glucose activation of mTOR is amino acid-dependent, whereas glucose-stimulated insulin secretion is not; 2) rapamycin at nanomolar concentrations completely inhibits glucose activation of mTOR but has no effect on glucose-stimulated insulin secretion; 3) mTOR activation is achieved at very low intracellular Ca^{2+} concentrations that are insufficient to support glucose-stimulated insulin secretion; and 4) glucose-stimulated activation of mTOR is partially blocked by the adenylyl cyclase inhibitor, MDL-12330A, whereas glucose-stimulated insulin secretion is unaltered.

Briaud et al. (29) have reported that glyburide at basal glucose concentrations did not stimulate S6K1 phosphorylation, and verapamil, an inhibitor of voltage-dependent Ca^{2+} channels, also failed to block glucose stimulation of S6K1 phosphorylation. An explanation for this lack of agreement with our findings is that these particular studies with glyburide were performed in a β -cell line, INS-1, as opposed to primary islets that exhibit a robust activation of S6K1 by glucose. It is also probable that cAMP production by INS-1 cells differs significantly from primary β -cells. Based on our studies with nifedipine alone and in combination with BAPTA to lower cytosolic Ca^{2+} concentrations by chelation, the inability of verapamil to produce partial inhibition of S6K1 phosphorylation by glucose may be due to the incomplete blockage of extracellular Ca^{2+} entry (compare lanes 3 and 4 in Fig. 7B).

THERAPEUTIC TARGETS OR SITES OF INTERVENTION

Although our understanding of the regulation of mTOR in β -cells is not yet complete, some important regulatory sites are described in Fig. 8. Because activation of β -cell mitochondria exerts a significant role in nutrient stimulation of mTOR, upregulation of glycolysis and/or provision of TCA intermediates represents a potential strategy to further enhance mTOR-mediated growth and proliferation. This may be the mechanism, in part, whereby the branched chain amino acid leucine, which is exclusively metabolized by the mitochondria, in combination with glucose participates in the activation of mTOR. Our studies have also identified K_{ATP} channels as possible therapeutic targets by their ability to regulate membrane depolarization and the influx of extracellular Ca^{2+} . It appears that the large oscillatory increases in cytosolic Ca^{2+} derived from only the extracellular media by metabolic inhibition of K_{ATP} channels are sufficient to facilitate both mTOR activation and insulin secretion. In contrast, the smaller increases in cytosolic Ca^{2+} achieved by the mobilization of only intracellular Ca^{2+} stores selectively activate mTOR, probably due to the very low cytosolic Ca^{2+} concentrations required to upregulate the mitochon-

dria, whereas higher cytosolic Ca^{2+} is necessary for glucose-stimulated insulin secretion. In our studies with primary islets, glyburide and agents that increase cAMP, including Exenatide and forskolin, were very effective in restoring full mTOR activation at suboptimal concentrations of glucose. These latter therapeutic sites would be advantageous under conditions in which the metabolism of glucose is impaired. The mobilization of intracellular stores of Ca^{2+} would be a site for therapeutic intervention under conditions in which sources of extracellular Ca^{2+} could not be utilized. In the event that adenylyl cyclase cannot function effectively to produce cAMP, cell-permeable analogs of cAMP may be utilized. In addition, Exenatide activation of the $\text{Gs}\beta\gamma$ portion of the GLP-1 receptor and stimulation of the PI3K and Akt pathway may be useful in overcoming a defect in adenylyl cyclase. Insulin, IGF-1, and other growth hormones such as GH and PTH (growth hormone and parathyroid hormone) may also exert a positive role in the upregulation of the mTOR pathway in conjunction with nutrient signaling.

CONCLUSION

Our findings suggest that the glucose-dependency of incretins to increase growth and proliferation may, in part, involve mTOR activation. This is attributed primarily to the function of the $\text{Gs}\alpha$ subunit of the GLP-1 receptor that activates adenylyl cyclases to produce cAMP. The ability of cAMP to mobilize intracellular Ca^{2+} stores enhances mitochondrial activation, which in turn can modulate K_{ATP} channels in the presence of the nutrients—glucose and amino acids—and thereby contributes to optimal activation of mTOR. mTOR activation is regulated by both basal and elevated glucose concentrations that are due, in part, to metabolic modulation of K_{ATP} channels. Our inability to demonstrate an effect of incretins at basal glucose may be due to the production of relatively low levels of cAMP in comparison to pharmacologic approaches employing forskolin (30). The potential significance of this signaling pathway is that mTOR remains activated during a continuous stimulation with elevated glucose concentrations and mediates DNA synthesis by primary islets in a rapamycin-sensitive manner. The ability of rapamycin to block basal glucose-stimulated ^3H -thymidine incorporation demonstrates a novel regulation of mTOR at low glucose concentrations. Based on indirect approaches with an adenylyl cyclase inhibitor, a significant component of glucose-mediated DNA synthesis may involve a role for cAMP. It is hoped that a more thorough knowledge of the mTOR signaling pathway will make it possible to use nutrients, incretins, and growth factors along with current diabetes treatments to circumvent points of dysfunction and promote β -cell survival, growth, and proliferation.

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