Ca^{2+}- and phospholipase D-dependent and -independent pathways activate mTOR signaling

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Received 11 June 2003; revised 15 July 2003; accepted 15 July 2003

First published online 30 July 2003

Edited by Lev Kisselev

Abstract The mammalian target of rapamycin (mTOR) promotes increased protein synthesis required for cell growth. It has been suggested that phosphatidic acid, produced upon activation of phospholipase D (PLD), is a common mediator of growth factor activation of mTOR signaling. We used Rat-1 fibroblasts expressing the α_{1A} adrenergic receptor to study if this G_q-coupled receptor uses PLD to regulate mTOR signaling. Phenylephrine (PE) stimulation of the α_{1A} adrenergic receptor induced mTOR autophosphorylation at Ser2481 and phosphorylation of two mTOR effectors, 4E-BP1 and p70 S6 kinase. These PE-induced phosphorylations were greatly reduced in cells depleted of intracellular Ca^{2+}. PE activation of PLD was also inhibited in Ca^{2+}-depleted cells. Incubation of cells with 1-butanol to inhibit PLD signaling attenuated PE-induced phosphorylation of mTOR, 4E-BP1 and p70 S6 kinase. By contrast, platelet-derived growth factor (PDGF)-induced phosphorylation of these proteins was not blocked by Ca^{2+} depletion or 1-butanol treatment. These results suggest that the α_{1A} adrenergic receptor promotes mTOR signaling via a pathway that requires an increase in intracellular Ca^{2+} and activation of PLD. The PDGF receptor, by contrast, appears to activate mTOR by a distinct pathway that does not require Ca^{2+} or PLD.

Key words: Mammalian target of rapamycin; Phospholipase D; Calcium; α_1 adrenergic receptor; Platelet-derived growth factor; Protein synthesis

1. Introduction

Activation of protein synthesis by growth factors is a complex process that involves phosphorylation of a number of components of the translational machinery. 4E-BP1 is a translation repressor that, when hypophosphorylated, binds to translation initiation factor eIF4E and prevents formation of a functional eIF4F complex. Treatment of cells with growth factors leads to the multiple phosphorylation of 4E-BP1 and its dissociation from eIF4E, thereby relieving the translational block [1]. Phosphorylation of the S6 protein in 40S ribosomal subunits is also associated with growth factor-induced activation of protein synthesis. The major kinase that phosphorylates S6 is p70 S6 kinase, which is activated by phosphorylation at multiple sites in response to growth factor treatment [2].

In many systems, growth factor-induced phosphorylation of 4E-BP1 and p70 S6 kinase is blocked by phosphatidylinositol (PI) 3-kinase inhibitors such as wortmannin and LY294002 and by the immunosuppressant rapamycin. Rapamycin, when bound to its intracellular receptor FKBP12, inhibits the function of the mammalian target of rapamycin (mTOR), a kinase whose catalytic domain resembles that of PI 3-kinase [3]. mTOR has been shown to phosphorylate 4E-BP1 and p70 S6 kinase at functionally important sites in vitro [4,5] and to undergo autophosphorylation at Ser2481 [6]. Signaling through mTOR is thought to be sensitive to nutrient levels and to the intracellular concentration of ATP [7–9].

Phosphorylation of 4E-BP1 and p70 S6 kinase has been proposed to be regulated by two parallel pathways that integrate growth factor signaling and nutrient sensing. Mitogen-activated signals are thought to be transduced through a PI 3-kinase-dependent pathway, while mTOR is thought to provide a permissive signal in the presence of sufficient nutrients that allows 4E-BP1 and p70 S6 kinase to respond to the PI 3-kinase signal [3]. Other models propose that mTOR itself is regulated by growth factor-generated PI 3-kinase signals [10]. In addition, it was recently proposed that phosphatidic acid, produced in response to growth factor activation of phospholipase D (PLD), binds to mTOR and is required for mTOR signaling to 4E-BP1 and p70 S6 kinase [11,12].

Pathological stimulation of α_1 adrenergic receptors by catecholamines plays an important role in the development of cardiac hypertrophy that can lead to heart failure [13]. Increased protein synthesis is required for cells to undergo hypertrophic growth. Since all three α_1 adrenergic receptors (α_{1A}, α_{1B}, and α_{1D}) are present in cardiac myocytes, we have utilized Rat-1 fibroblasts that stably express the α_{1A} adrenergic receptor to investigate how this receptor controls protein synthesis. We previously demonstrated that activation of the α_{1A} adrenergic receptor in these cells stimulates protein synthesis and induces 4E-BP1 phosphorylation and p70 S6 kinase activation [14]. However, unlike some growth factors such as platelet-derived growth factor (PDGF), α_{1A} adrenergic receptor signaling to 4E-BP1 and p70 S6 kinase does not require activation of PI 3-kinase [15], but is instead mediated

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Abbreviations: PE, phenylephrine; mTOR, mammalian target of rapamycin; PI, phosphatidylinositol; PLD, phospholipase D; PKC, protein kinase C; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HEK, human embryonic kidney

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doi:10.1016/S0014-5793(03)00816-0

by a Ca^{2+}-dependent pathway [14]. In the course of studying this Ca^{2+}-dependent pathway, we found that there are distinct differences in the mechanisms used by the α_{1A} adrenergic receptor and the PDGF receptor to regulate mTOR signaling. We determined that stimulation of both receptors affects the activity of mTOR, as reflected by autophosphorylation of Ser2481. We next evaluated whether Ca^{2+} and PLD play a role in mediating this effect. Our findings suggest that stimulation of mTOR signaling by the α_{1A} adrenergic receptor, but not the PDGF receptor, requires an increase in intracellular Ca^{2+} and activation of PLD.

2. Materials and methods

2.1. Materials

Phenylephrine, PDGF A/B, 1-butanol, BAPTA-AM and A23187 were from Sigma (St. Louis, MO, USA). [^{3}H]Palmitic acid (30–60 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA). Rapamycin, LY294002 and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem (San Diego, CA, USA). Phospho-specific antibodies and antibody to total mTOR were from Cell Signaling Technology (Beverly, MA, USA). Antibodies to total 4E-BP1 and total p70 S6 kinase were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

Rat-1 fibroblasts stably transfected with the human α_{1A} adrenergic receptor [16] were maintained in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, USA) containing antibiotics and 10% fetal bovine serum (Sigma). Cells were incubated in serum-free medium for 16–18 h before treatments. For experiments involving Ca^{2+}, cells were preincubated for 1 h in high-salt glucose buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM $MgSO_4$, 1 mM KH_2PO_4 and 10 mM glucose) plus either 2 mM EGTA or 1 mM Ca^{2+} before adding agonist to the buffer.

2.3. Immunoblotting

Cell lysates were prepared in buffer containing 50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin and leupeptin. Proteins were subjected to SDS gel electrophoresis and Western blotting as previously described [15]. The integrated density of bands was quantified using Scion Image Beta 4.0.2. The numerical values under the Western blots represent a ratio obtained by dividing the integrated density of bands in the upper panels by that of the corresponding bands in the lower panels.

2.4. PLD assay

Cells were labeled with 2 μCi/ml of [^{3}H]palmitic acid in growth medium for 48 h. The labeling medium was replaced with either serum-free medium or high-salt glucose buffer 1 h prior to agonist treatments. 0.3% 1-butanol was added just before the agonist. The cells were extracted with methanol and [^{3}H]phosphatidylbutanol was measured as previously described [17].

3. Results and discussion

3.1. α_{1A} adrenergic receptor stimulation promotes mTOR autophosphorylation

Although growth factors can cause large increases in 4E-BP1 phosphorylation and p70 S6 kinase activity, increases in mTOR kinase activity measured in vitro are usually relatively small. Phosphorylation of Ser2481 in wild-type mTOR, but not a kinase-dead mutant, was readily detected in cells, suggesting that Ser2481 is an autophosphorylation site that can serve as a reporter of in vivo mTOR kinase activity [6]. We therefore used a phospho-specific antibody and Western blotting to assess changes in mTOR Ser2481 phosphorylation after exposing Rat-1 cells to phenylephrine (PE), an α_{1A} adrenergic receptor agonist. mTOR in serum-starved cells exhibited a basal level of Ser2481 phosphorylation (Fig. 1A, top blot). Stimulation of the α_{1A} adrenergic receptor led to an increase in phosphorylation that was nearly maximal at 10 min and persisted up to 30 min. A comparable increase in Ser2481 phosphorylation was seen after serum stimulation of human embryonic kidney (HEK) 293 cells [6]. The membrane was reprobed with a general mTOR antibody to confirm that similar amounts of the protein were present in each lane (Fig. 1A, bottom blot).

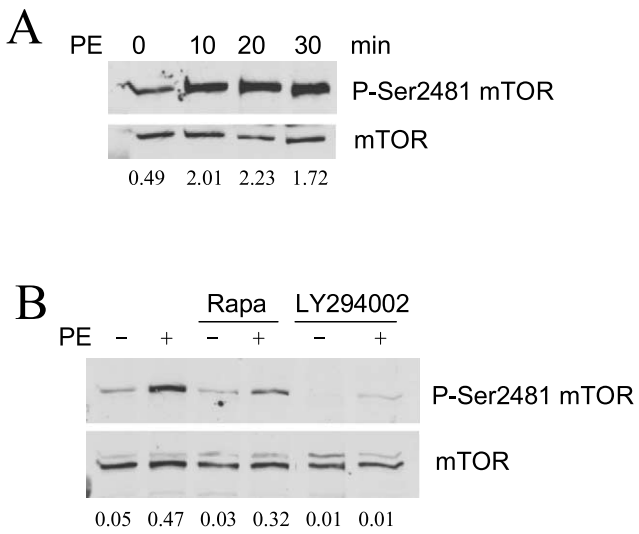

Fig. 1. PE-induced mTOR phosphorylation is blocked by rapamycin and LY294002. A: Cells were treated with 10 μM PE for the indicated times. Equal amounts of cell lysate protein were analyzed on a Western blot probed sequentially with antibodies to phospho-Ser2481 mTOR (top blot) and total mTOR (lower blot). B: Cells were pretreated for 30 min with vehicle, 20 nM rapamycin or 50 μM LY294002, followed by stimulation with or without 10 μM PE for 20 min. Cell lysate proteins were analyzed on a Western blot probed sequentially with antibodies to phospho-Ser2481 mTOR (top blot) and total mTOR (lower blot). The experiments were done at least three times and representative results are shown.

In a prior study, we showed that rapamycin and LY294002 interfere with α_{1A} adrenergic receptor signaling to 4E-BP1 and p70 S6 kinase [14]. The effect of these two inhibitors on α_{1A} adrenergic receptor-induced mTOR phosphorylation was examined by incubating serum-starved cells with rapamycin or LY294002 prior to stimulation with PE. PE-induced phosphorylation of mTOR at Ser2481 was attenuated in the presence of rapamycin (Fig. 1B, top blot). LY294002 was an even more effective inhibitor of mTOR phosphorylation: the basal phosphorylation of Ser2481 was reduced to an undetectable level, and mTOR autophosphorylation in response to PE treatment was almost completely blocked (Fig. 1B, top blot).

In contrast to our results, Peterson et al. detected no significant change in Ser2481 phosphorylation after treatment of cells with rapamycin [6]. This discrepancy might be due to differences in the order of addition of drug and agonist: in Ref. [6] rapamycin was added to cells that were already growing in serum, whereas we added the drug to serum-starved cells prior to stimulation with agonist. It is possible that rapamycin does not effectively reverse Ser2481 phosphorylation, but is effective at preventing agonist-induced mTOR autophosphorylation. Similar to our results using LY294002,

wortmannin was also reported to significantly inhibit mTOR Ser2481 autophosphorylation [6]. Although LY294002 and wortmannin are best known as PI 3-kinase inhibitors, these two compounds have also been shown to inhibit mTOR kinase activity [18]. PE treatment of Rat-1 cells does not activate PI 3-kinase [15], so the inhibitory effect of LY294002 on mTOR phosphorylation in response to PE treatment is presumably due to a direct effect on mTOR and not to PI 3-kinase inhibition.

3.2. PE-induced mTOR phosphorylation is Ca^{2+}-dependent

Like all G_q-coupled receptors, stimulation of the α_{1A} adrenergic receptor leads to an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [15]. We reported that pretreatment of Rat-1 cells with BAPTA-AM to chelate intracellular Ca^{2+} inhibits PE-induced 4E-BP1 phosphorylation and p70 S6 kinase activation [14]. In addition, raising the $[Ca^{2+}]_i$ with the Ca^{2+} ionophore A23187 increased the phosphorylation of 4E-BP1 and activated p70 S6 kinase [14]. To examine the role of Ca^{2+} in α_{1A} adrenergic receptor signaling to mTOR, serum-starved cells were preincubated with or without BAPTA-AM prior to stimulation with PE. BAPTA-AM strongly inhibited PE-induced mTOR phosphorylation (Fig. 2A, top blot). In addition, raising the $[Ca^{2+}]_i$ with A23187 stimulated mTOR phosphorylation as much as PE treatment did (Fig. 2A, top blot).

Treatment of Rat-1 cells with PDGF, unlike PE, causes a substantial increase in PI 3-kinase activity [15]. PDGF also stimulates 4E-BP1 phosphorylation and p70 S6 kinase activation in a rapamycin-sensitive manner, indicating a requirement for mTOR [14]. We asked whether the PDGF receptor also requires Ca^{2+} to stimulate mTOR signaling. Rat-1 cells were treated with PE or PDGF in buffer containing either Ca^{2+} or EGTA; the latter condition has been shown to completely abolish the PE-induced increase in $[Ca^{2+}]_i$ [15]. In the presence of Ca^{2+}, both PE and PDGF induced a robust increase in mTOR phosphorylation, although the effect with PDGF was stronger (Fig. 2B, top panel). Interestingly, PE-induced Ser2481 phosphorylation was strongly inhibited in the presence of EGTA, whereas the response to PDGF was largely unaffected by Ca^{2+} depletion (Fig. 2B, top blot). Together, these results suggest that a rise in $[Ca^{2+}]_i$ is required for PE-induced, but not PDGF-induced, mTOR phosphorylation. In addition, high $[Ca^{2+}]_i$ alone is sufficient to trigger mTOR Ser2481 phosphorylation.

Using phospho-specific antibodies and Western blotting, we also compared the Ca^{2+} dependence of phosphorylation of 4E-BP1 and p70 S6 kinase in response to PE and PDGF. mTOR has been shown to directly phosphorylate p70 S6 kinase at Thr389 [5] and to promote 4E-BP1 phosphorylation at Ser64 [19]. In the presence of Ca^{2+}, treatment with either PE or PDGF stimulated the phosphorylation of 4E-BP1 (Fig. 2C, top blot) and p70 S6 kinase (Fig. 2C, third blot). Similar to what was seen with mTOR autophosphorylation, PDGF evoked stronger responses than PE. In addition, PE-induced phosphorylation of Ser64 4E-BP1 and Thr389 p70 S6 kinase was strongly inhibited in the presence of EGTA, whereas the effect of PDGF was still intact in Ca^{2+}-depleted cells (Fig. 2C, top and third blots). The membranes were reprobed for total 4E-BP1 (Fig. 2C, second blot) and p70 S6 kinase (Fig. 2C, bottom blot) to demonstrate that each lane contained similar amounts of each protein. These results indicate that autophos-

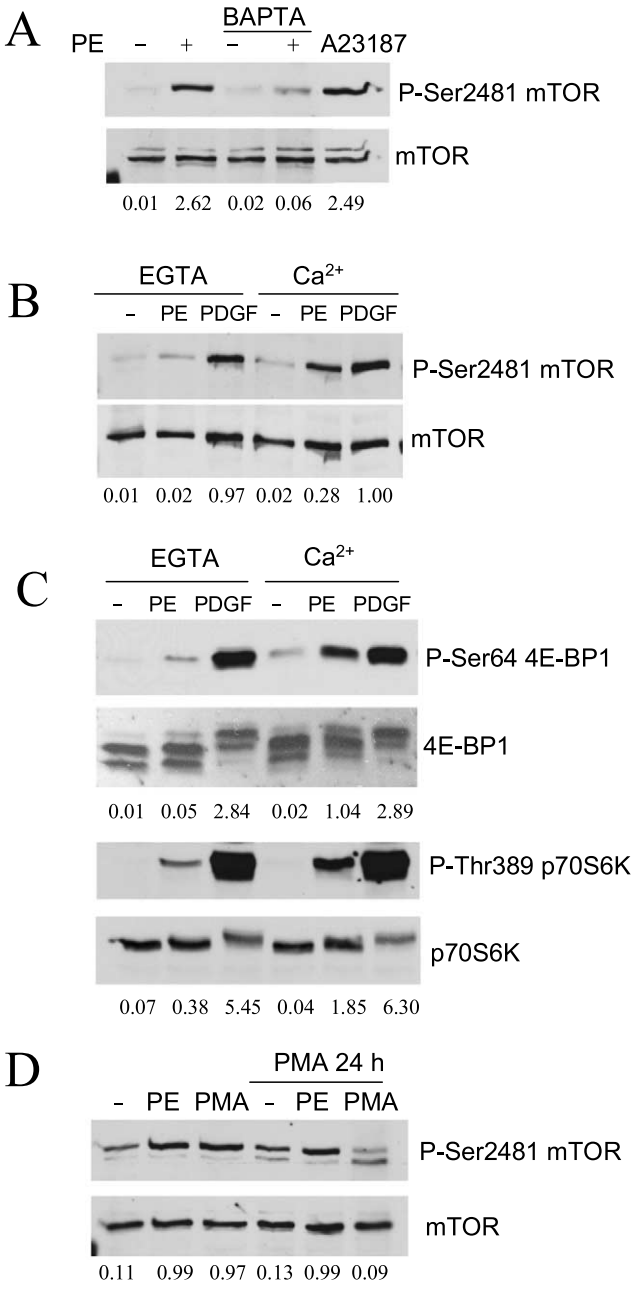

Fig. 2. Effect of $[Ca^{2+}]_i$ on PE-induced phosphorylation of mTOR, 4E-BP1 and p70 S6 kinase. A: Cells were pretreated for 30 min with 10 μM BAPTA-AM or vehicle and then stimulated for 20 min with or without 10 μM PE. In the right lane, cells were treated with 10 μM A23187 for 20 min. Phospho-Ser2481 mTOR (top blot) and total mTOR (lower blot) were detected on a Western blot. B: Cells were preincubated in high-salt glucose buffer with EGTA or Ca^{2+} (see Section 2.2), then stimulated for 20 min with or without 10 μM PE or 25 ng/ml PDGF. Equal amounts of cell lysate protein were analyzed on a Western blot probed sequentially with antibodies to phospho-Ser2481 mTOR (top blot) and total mTOR (bottom blot). C: Cells were treated as in B. Cell lysate proteins were analyzed on Western blots to detect phospho-Ser64 4E-BP1 (numbering is based on the sequence of the rat protein) and total 4E-BP1 (top two blots), and phospho-Thr389 p70 S6 kinase and total p70 S6 kinase (bottom two blots). Total 4E-BP1 appears as multiple bands due to migration differences of differentially phosphorylated species. D: Cells were preincubated in serum-free medium with or without 100 nM PMA for 24 h, followed by treatment with or without 10 μM PE or 100 nM PMA for 20 min. Phospho-Ser2481 mTOR (top blot) and total mTOR (lower blot) were detected on a Western blot. The experiments were done three times and representative results are shown.

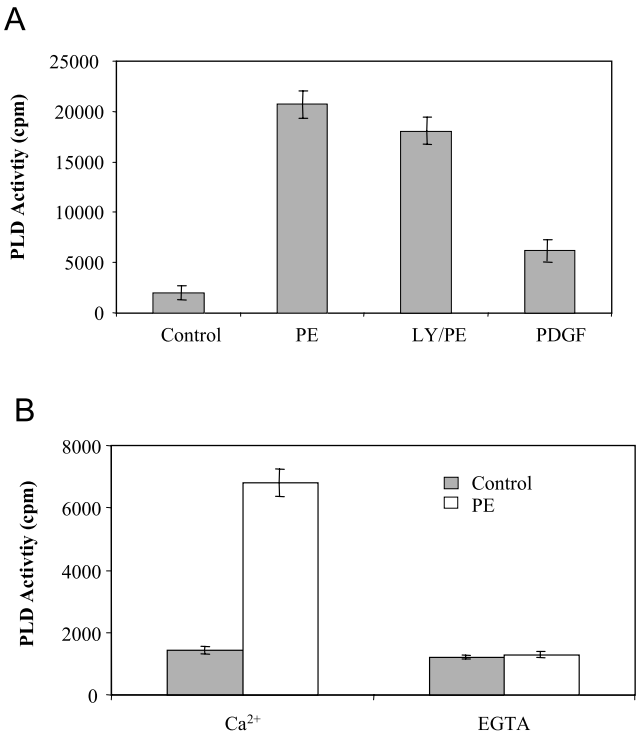

Fig. 3. Effect of LY294002 and $[Ca^{2+}]_i$ on PE-induced PLD activation. Cells were labeled with [^{3}H]palmitate in normal growth medium for 48 h. After treatments, cells were extracted with methanol and [^{3}H]phosphatidylbutanol levels were measured (see Section 2.4). A: Labeled cells were placed into serum-free medium for 30 min, and then were incubated for 30 min with or without 50 μM LY294002. Then the cells were stimulated for 20 min with or without 10 μM PE or 25 ng/ml PDGF. Data shown are means ± standard error ($n = 5$ for Control and PE; $n = 3$ for LY/PE and PDGF). B: Labeled cells were incubated for 1 h in high-salt glucose buffer with Ca^{2+} or EGTA (see Section 2.2), then stimulated for 20 min with or without 10 μM PE. Data shown are means ± standard error ($n = 4$).

phorylation of mTOR and mTOR signaling to downstream effectors in response to α_{1A} adrenergic receptor stimulation is Ca^{2+}-dependent. PDGF apparently uses a different mechanism to activate mTOR signaling that does not require an increase in $[Ca^{2+}]_i$.

3.3. PE-induced mTOR phosphorylation is not dependent on PKC

The increase in $[Ca^{2+}]_i$ and production of diacylglycerol following stimulation of G_q-coupled receptors leads to activation of Ca^{2+}- and diacylglycerol-dependent isoforms of protein kinase C (PKC) [13]. We investigated the role of these enzymes in α_{1A} adrenergic receptor-mediated phosphorylation of mTOR by treating cells with or without PMA for 24 h to down-regulate PKCs prior to challenge with an agonist. Treatment of control cells with either PE or PMA for 20 min induced the phosphorylation of Ser2481 mTOR (Fig. 2D, upper blot). In cells pretreated for 24 h with PMA, phosphorylation of mTOR by a subsequent challenge with PMA was abolished, but there was no effect on PE-induced phosphorylation of the protein (Fig. 2D, upper blot). Thus, phosphorylation of mTOR promoted by the α_{1A} adrenergic receptor does not require PMA-sensitive PKCs. This result is consistent with our observations that PE stimulation of 4E-BP1 phosphorylation [14] and p70 S6 kinase activation [15] is also independent of PMA-sensitive PKCs.

3.4. PE activation of PLD is Ca^{2+}-dependent

It is well known that increases in $[Ca^{2+}]_i$ in response to hormone stimulation or ionophore treatment can lead to activation of PLD [20]. Using an in vivo assay, we first tested if treatment of cells with agonists in serum-free medium increases PLD activity. PE treatment activated PLD approximately 10-fold (Fig. 3A). This response was comparable to that achieved using PMA, which is one of the most potent PLD activators known (data not shown). In contrast, PDGF treatment stimulated PLD activity only about three-fold (Fig. 3A).

We next examined whether the PE-induced increase in PLD activity is dependent on $[Ca^{2+}]_i$. PE treatment of cells in Ca^{2+}-containing buffer stimulated PLD activity approximately four-fold, whereas Ca^{2+} depletion with EGTA completely abol-

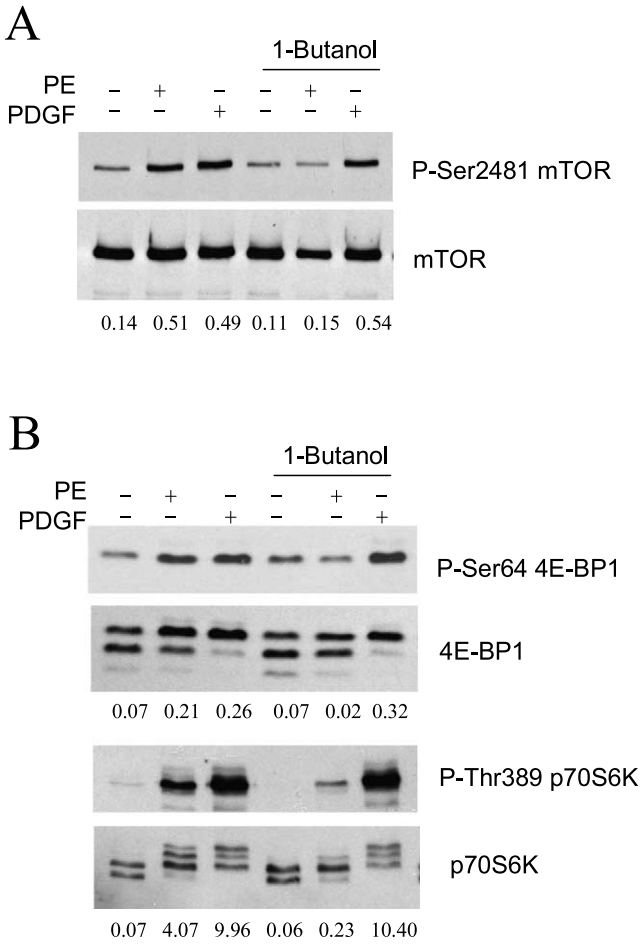

Fig. 4. Effect of 1-butanol on PE-induced phosphorylation of mTOR, 4E-BP1 and p70 S6 kinase. Serum-starved cells were preincubated with or without 0.3% 1-butanol for 30 min, then stimulated for 20 min with 10 μM PE or 25 ng/ml PDGF. A: Equal amounts of cell lysate protein were analyzed on a Western blot probed sequentially with antibodies to phospho-Ser2481 mTOR (top blot) and total mTOR (bottom blot). B: Cell lysate proteins were analyzed on Western blots to detect phospho-Ser64 4E-BP1 and total 4E-BP1 (top two blots), and phospho-Thr389 p70 S6 kinase and total p70 S6 kinase (bottom two blots). Total 4E-BP1 and total p70 S6 kinase appear as multiple bands due to migration differences of differentially phosphorylated species. The experiment was done three times with similar results.

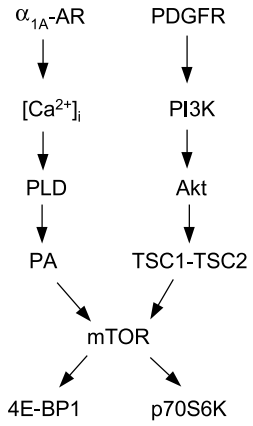

Fig. 5. Model for two pathways regulating mTOR. The α_{1A} adrenergic receptor (α_{1A}-AR) activates a pathway that requires an increase in $[Ca^{2+}]_i$, activation of PLD, and accumulation of phosphatidic acid (PA) to induce mTOR activation and phosphorylation of 4E-BP1 and p70 S6 kinase (p70S6K). The PDGF receptor (PDGFR) activates PI 3-kinase (PI3K) and Akt, which phosphorylates TSC1–TSC2 to activate mTOR signaling [10].

ished this response (Fig. 3B). Malik and coworkers reported a similar result using this cell system [21]. In addition, these authors found that PE activation of PLD is independent of PKC [21]. Together, these results indicate that PE activation of PLD, like mTOR autophosphorylation, is Ca^{2+}-dependent but PKC-independent.

3.5. 1-Butanol inhibits PE-stimulated mTOR phosphorylation

Phosphatidic acid, a lipid second messenger produced by PLD, has been proposed to be a critical component for mTOR signaling [11]. PE-induced PLD activation was not blocked in cells pretreated with LY294002 (Fig. 3A), suggesting that PLD might indeed act upstream of mTOR, but not vice versa. To test the hypothesis that PLD and mTOR act in a common signaling pathway, cells were pretreated with 1-butanol prior to stimulation with agonist. Through a transphosphatidylation reaction, 1-butanol prevents the production of phosphatidic acid. PE-induced Ser2481 mTOR phosphorylation was completely blocked in the presence of 1-butanol, whereas the PDGF-stimulated increase was largely unaffected (Fig. 4A, top blot). Likewise, pretreatment with 1-butanol blocked PE-induced phosphorylation of Ser64 on 4E-BP1 (Fig. 4B, top blot) and Thr389 on p70 S6 kinase (Fig. 4B, third blot), but increased phosphorylation at these sites in response to PDGF was still intact.

3.6. Two distinct pathways control mTOR signaling

Chen and coworkers recently reported that serum treatment of HEK 293 cells led to the PLD-dependent accumulation of phosphatidic acid, which was required for 4E-BP1 phosphorylation and p70 S6 kinase activation [11]. They speculated that this might be a common pathway used by mitogens to activate mTOR signaling [11,12]. However, the data presented here suggest that PLD activation is used by only a subset of growth factors to regulate the mTOR pathway.

Our data indicate that PE stimulation of the α_{1A} adrenergic receptor activates a signaling pathway that includes an increase in $[Ca^{2+}]_i$, activation of PLD, autophosphorylation of mTOR, and phosphorylation of the mTOR effectors 4E-BP1 and p70 S6 kinase (Fig. 5). These results support the conclusion of Chen and coworkers [11] that PLD can regulate mTOR. We speculate that G protein-coupled receptors that mobilize intracellular Ca^{2+} but do not activate PI 3-kinase might require the PLD-dependent mechanism to regulate mTOR. A selective requirement for PLD to activate mTOR signaling by G protein-coupled receptors might allow development of drugs to selectively block pathological activation of mTOR by this receptor family.

By contrast, even though the PDGF receptor activates PLD, it appears to use a distinct pathway that does not require an increase in $[Ca^{2+}]_i$ or PLD activity to activate mTOR signaling. One possibility is that this receptor also activates diacylglycerol kinase to produce phosphatidic acid; this reaction would not be inhibited by 1-butanol. Alternatively, the PDGF receptor, and perhaps other tyrosine kinase receptors, might require the activation of PI 3-kinase to stimulate mTOR signaling (Fig. 5). Recent data suggest that PI 3-kinase and its downstream effector, the protein kinase Akt, control mTOR through the TSC1-TSC2 tumor suppressor complex [10]. According to a model based on these data, in the absence of growth factors the TSC1–TSC2 complex is hypophosphorylated and inhibits mTOR. Growth factor activation of PI 3-kinase/Akt results in the phosphorylation and inhibition of TSC1-TSC2, leading to derepression of mTOR, which can then phosphorylate 4E-BP1 and p70 S6 kinase [10]. In addition to a direct effect of phosphatidic acid on mTOR [11], one might imagine that mTOR activation by the α_{1A} adrenergic receptor would also require inhibition of TSC1–TSC2. If so, the kinase that phosphorylates TSC1-TSC2 would be distinct from Akt, since the α_{1A} adrenergic receptor does not activate Akt [15]. Indeed, we have found that treatment of Rat-1 cells with PE does not induce the phosphorylation of TSC2 at Thr1462, whereas PDGF causes an increase in phosphorylation at this site (data not shown). Thr1462 on TSC2 is thought to be a functionally important Akt phosphorylation site [10]. Future experiments are aimed at testing whether agonists that regulate mTOR by the PLD pathway promote phosphorylation of TSC1–TSC2 at other sites that regulate the function of the complex.

Acknowledgements: This work was supported by NIH R01 DK62722 and by a Department of Veterans Affairs Medical Research merit review grant (to R.Z.L.).

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