The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle

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Introduction

This study investigates the role phospholipase D has when mechanically activated by muscle stretch, and if that effect is mediated by mTOR.

Conclusions

There is an association with increased phosphatidic acid and increased mTOR downstream signaling - implying, phosphatidic acid plays a key role in mTOR mediated cell growth.

Phospholipase's production of phosphatidic acid is a specific mechanosensation mechanism that is independent of other cell stress signaling mechanisms.

Amendments

Study Design & Need to Know Information

Researchers used whole muscles removed from mice and mounted them to allow the muscles to be stretched (to induce a mechanical stretch). The muscles were also exposed to a variety of drugs/inhibitors (described in each figure notes) to block the activity of key enzymes to study the effect of muscle stretch on mTOR signaling.

mTOR: Mammalian Target of Rapamycin; the master cell growth enzyme that phosphorylates (activates) the protein **p70** to enhance muscle cell growth.

PLD: Phospholipase D; this enzyme translates stretch (mechanosensation) on the muscle into a chemical signal by producing **phosphatidic acid**, which binds mTOR (supposedly), and activates mTOR signaling.

The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle

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Signaling by the mammalian target of rapamycin (mTOR) has been reported to be necessary for mechanical load-induced growth of skeletal muscle. The mechanisms involved in the mechanical activation of mTOR signaling are not known, but several studies indicate that a unique [phosphotidylinositol-3-kinase (PI3K)- and nutrient-independent] mechanism is involved. In this study, we have demonstrated that a regulatory pathway for mTOR signaling that involves phospholipase D (PLD) and the lipid second messenger phosphatidic acid (PA) plays a critical role in the mechanical activation of mTOR signaling. First, an elevation in PA concentration was sufficient for the activation of mTOR signaling. Second, the isozymes of PLD (PLD1 and PLD2) are localized to the z-band in skeletal muscle (a critical site of mechanical force transmission). Third, mechanical stimulation of skeletal muscle with intermittent passive stretch *ex vivo* induced PLD activation, PA accumulation, and mTOR signaling. Finally, pharmacological inhibition of PLD blocked the mechanically induced increase in PA and the activation of mTOR signaling. Combined, these results indicate that mechanical stimuli activate mTOR signaling through a PLD-dependent increase in PA. Furthermore, we showed that mTOR signaling was partially resistant to rapamycin in muscles subjected to mechanical stimulation. Because rapamycin and PA compete for binding to the FRB domain on mTOR, these results suggest that mechanical stimuli activate mTOR signaling through an enhanced binding of PA to the FRB domain on mTOR.

mechanical load | skeletal muscle growth | exercise | stretch | atrophy

Mechanical loads play a central role in the regulation of skeletal muscle mass; however, the mechanisms involved in converting mechanical signals into the molecular events that control this process have not been defined (1, 2). Recent studies on this topic have focused on a signaling network that is regulated by a protein kinase called the mammalian target of rapamycin (mTOR) (3–5). Interest in mTOR signaling was initially motivated by studies that suggested that this protein was the master regulator of a signaling network that controls cell growth (6, 7). One of the most intensely studied proteins in the mTOR signaling network is the ribosomal S6 kinase ($p70^{S6k}$), and measurements of $p70^{S6k}$ phosphorylation on the threonine 389 residue [$p70^{S6k}$ (389)] are commonly used as a readout of mTOR signaling (6, 8, 9).

Mechanical loading of skeletal muscle has been shown to induce $p70^{S6k}$ (389) phosphorylation through a rapamycin-sensitive mechanism, implicating a role for mTOR in this pathway (5, 10). Furthermore, the mechanical activation of $p70^{S6k}$ phosphorylation correlates with the extent of the concomitant growth response, and inhibiting mTOR with rapamycin prevents the mechanical-load-induced growth (3–5, 11) Taken together, these observations indicate that the activation of mTOR signaling is an essential step for converting mechanical signals into a growth response.

To date, the mechanisms involved in the mechanical activation of mTOR signaling have not been defined. However, the mechanisms for the activation of mTOR signaling in response to other forms of growth-promoting stimuli, such as nutrients and growth factors, have been fairly well established to involve a wortmannin-sensitive mechanism [i.e., phosphotidylinositol-3-kinase (PI3K)-dependent] (8, 12). Furthermore, the presence of exogenous nutrients is required for the activation of mTOR signaling by growth factors (12). Conversely, mechanical stimuli have been shown to activate mTOR signaling through a wortmannin-independent mechanism that does not require exogenous nutrients (5, 13). These findings indicate that mechanical stimuli use a unique (PI3K- and nutrient-independent) mechanism to activate mTOR signaling.

Recently, a regulatory mechanism of mTOR signaling involving phospholipase D (PLD) and the lipid second messenger phosphatidic acid (PA) was described (8, 14–16). In this study, we examined the potential role of this pathway in the mechanical activation of mTOR signaling, and our results indicate that a PLD-dependent increase in PA is an essential step in the unique mechanism through which mechanical stimuli activate mTOR signaling.

Results

PA Is Sufficient for the Activation of mTOR Signaling. To determine whether an elevation in PA was sufficient for the induction of mTOR signaling in skeletal muscle, mouse extensor digitorum longus (EDL) muscles were incubated in an *ex vivo* organ culture system with a PA phosphatase inhibitor propranolol (17). As shown in Fig. 1, incubating muscles with propranolol promoted an increase in the concentration of PA ([PA]) and an increase in $p70^{S6k}$ (389) phosphorylation. When muscles were incubated with rapamycin, $p70^{S6k}$ (389) phosphorylation was abolished in both control and propranolol-treated muscles (Fig. 1*B*). These results indicate that an elevation in [PA] leads to an activation of mTOR signaling in skeletal muscle.

Mechanical Stimulation Induces PLD Activity and PA Accumulation. To begin testing the role of PLD and PA in the mechanical activation of mTOR signaling, we used an *ex vivo* model for mechanically stimulating the EDL muscle (5). This model utilizes intermittent passive stretch as a source of mechanical stimulation and has been shown to activate mTOR-dependent signaling to $p70^{S6k}$ (389) (5). Measurements at various time points (15, 30, 60, and 90 min) after the initiation of the mechanical stimulation revealed a transient increase (15 min) in PLD activity and a sustained (15–90 min) increase in [PA] (Fig.

Conflict of interest statement: No conflicts declared.

Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl) ester; DAG, diacylglycerol; EDL, extensor digitorum longus; iCa^{2+}, intracellular Ca^{2+}; mTOR, mammalian target of rapamycin; PA, phosphatidic acid; [PA], PA concentration; PLC, phospholipase C; PLD, phospholipase D; PI3K, phosphotidylinositol-3-kinase; PKB, protein kinase B; $p70^{S6k}$, ribosomal S6 kinase.

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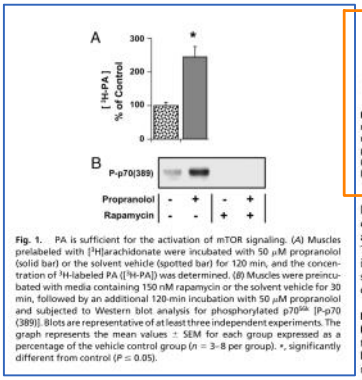

Fig. 1. PA is sufficient for the activation of mTOR signaling. (*A*) Muscles prelabeled with [³H]arachidonate were incubated with 50 μM propranolol (solid bar) or the solvent vehicle (spotted bar) for 120 min, and the concentration of ³H-labeled PA ([³H-PA]) was determined. (*B*) Muscles were preincubated with media containing 150 nM rapamycin or the solvent vehicle for 30 min, followed by an additional 120-min incubation with 50 μM propranolol and subjected to Western blot analysis for phosphorylated $p70^{S6k}$ [P-p70 (389)]. Blots are representative of at least three independent experiments. The graph represents the mean values ± SEM for each group expressed as a percentage of the vehicle control group ($n = 3$–8 per group). *, significantly different from control ($P \leq 0.05$).

Fig. 3. Muscles were preincubated with media containing 1–10 mM neomycin or the solvent vehicle (Neomycin −) for 30 min and then subjected to 90 min of mechanical stretch (Stretch −) or control conditions (Stretch −). Samples were subjected to Western blot analysis for phosphorylated $p70^{S6k}$ [P-p70 (389)] (*A*) or phosphorylated p38 and JNK2 (*B*). Blots are representative of at least three independent experiments.

2). It is worth noting that the time course of the mechanically induced increase in [PA] was significantly correlated ($R^2 = 0.78$) with the time course that we reported for mechanical activation of mTOR signaling to $p70^{S6k}$ (389) in this model (5).

Neomycin Inhibits the Mechanical Activation of mTOR Signaling but Not p38 or JNK. To determine whether PLD was required for the mechanical activation of mTOR signaling, muscles were incubated with the PLD/phospholipase C (PLC) inhibitor neomycin. In the presence of neomycin, basal $p70^{S6k}$ (389) phosphorylation was elevated to a similar degree with all doses, and the mechanically induced increase in $p70^{S6k}$ (389) phosphorylation was inhibited in a dose-dependent manner (Fig. 3*A*). Specifically, 10 mM neomycin completely blocked the mechanically induced increase in $p70^{S6k}$ (389) phosphorylation, because there was no significant difference between control and mechanically stimulated muscles at this dose. Ten millimolar neomycin, however, did not inhibit mechanically induced signaling to the stress-activated protein kinases (SAPKs) p38 and JNK2 (Fig. 3*B*). These results demonstrate the specificity of neomycin and indicate that mechanical stimuli activate at least two major signaling pathways (mTOR and SAPKs) that diverge upstream of the neomycin-sensitive mechanism.

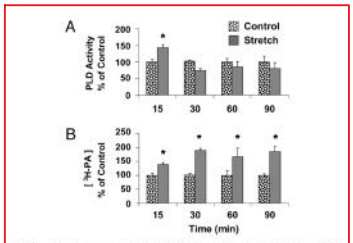

Fig. 2. Muscles were prelabeled with [³H]arachidonate, subjected to 15–90 min of mechanical stretch or control conditions, and measurements were made of PLD activity (*A*) or the concentration of ³H-labeled PA ([³H-PA]) (*B*) at the indicated time points. Graphs represent the mean values ± SEM for each group expressed as a percentage of the time-matched control ($n = 3$–4 per group). *, significantly different from time-matched control, ($P = 0.05$).

Inhibitors of Intracellular (i)Ca^{2+}, PKC, and PLC Do Not Prevent the Mechanical Activation of mTOR Signaling. Neomycin is a polycationic aminoglycoside that binds phosphatidylinositol 4,5-bisphosphate (PIP2) with a high affinity (18). It has been reported that PIP2 is a critical cofactor in the regulation of PLD and that neomycin inhibits PLD activity by binding to PIP2 (19, 20). Binding of neomycin to PIP2 has also been reported to inhibit PLC-dependent signaling events by preventing the conversion of PIP2 to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (21, 22). IP3 and DAG are secondary messengers that can promote the release of calcium from intracellular stores (iCa^{2+}) and activate PKC, respectively (23). To determine whether neomycin prevented the mechanical activation of mTOR signaling by blocking these PLC-dependent signaling events, pharmacological inhibitors of iCa^{2+}, PKC, and PLC were used.

The role of iCa^{2+} in the mechanical activation of mTOR signaling was assessed by using the intracellular calcium chelator 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl) ester (BAPTA-AM). BAPTA-AM was found to block the increases in protein kinase B (PKB) and p38 phosphorylation in muscles incubated with the calcium ionophore A23187, thus verifying that BAPTA-AM inhibited iCa^{2+}-induced signaling events. BAPTA-AM, however, did not inhibit the mechanically induced signaling to $p70^{S6k}$ (389) (Fig. 4*A*). This observation indicates that iCa^{2+} is not involved in mechanical activation of mTOR signaling.

The role of PKCs in the mechanical activation of mTOR signaling was assessed by using the PKC-specific inhibitor bisindolylmaleimide I (BIM). BIM was found to block the increase in $p70^{S6k}$ (389) phosphorylation in muscles stimulated with the PKC agonist TPA, thus verifying that BIM inhibited PKC-dependent signaling. BIM, however, did not inhibit the mechanically induced signaling to $p70^{S6k}$ (389) (Fig. 4*B*). This finding indicates that PKCs are not involved in the mechanical activation of mTOR signaling.

The role of PLC in the mechanical activation of mTOR signaling was assessed by using the PLC-specific inhibitor U73122. U73122 was found to block the increase in $p70^{S6k}$ (389) phosphorylation in muscles stimulated with the PLC agonist lysophosphatidic acid, thus verifying that U73122 inhibited PLC-dependent signaling. U73122, however, did not inhibit the mechanically induced signaling to $p70^{S6k}$ (389) (Fig. 4*C*). Taken together, the results from the above experiments indicate that PLC-, iCa^{2+}-, and PKC-dependent signaling events are not

Figure 1: The researchers have put a whole muscle (taken from a mouse) and dipped it into a solution. That solution is different, depending what they are trying to accomplish. Propranolol increases phosphatidic acid production. Rapamycin inhibits mTOR. Then, they are measuring the amount of phosphorylated p70 (which is phosphorylated by mTOR). If you're confused, please read the "Study Design & Need to Know Information" I have provided above. 1A shows the amount of phosphatidic acid produced. 1B shows the amount of phospho-p70 protein (darker smudge/line is more).

Primary Results:
- With the addition of only propranolol, phosphatidic acid production increases, and so does the amount of phospho-p70.
- With the addition of rapamycin, there is no phospho-p70 present.

Take Away: p70 is phosphorylated (activated) with the increase in phosphatidic acid, but this effect is eliminated by rapamycin, implying this phosphatidic acid effect is mTOR dependent.

Figure 3: The researchers are either not inducing or inducing a stretch (Stretch: - / +) onto the muscles and/or adding neomycin (a drug that inhibits phospholipase D ((PLD)) - see notes in "Study Design") at varying concentrations (10mM being the highest), and then measuring the amount of phosphorylated (activated) of p70 (see notes in "Study Design"), as well as p38 and JNK2, which are stress signalers (this was done to show the PLD inhibitor is specific to mechanosensation through p76 and not other stress signaling, as p38 and JNK2 are stress signalers, but not mechanostress signaling).

Primary Results
- Greater concentrations of neomycin elicit lower and lower phospho-p70.
- Stretch induces greater phospho-p70, but is inhibited by neomycin.
- Neither stretch nor neomycin have an effect when measuring mechanosense independent pathway signalers (p38, JNK2).

Take Away: Phospho-p70 is affected by the inhibition of Phospholipase (decreasing its prevalence), and enhanced by stretch, and this effect is specific to mechanosensation/stress pathway.

Figure 2: The researchers have placed a full muscle of a mouse into an apparatus by which they can keep the muscle tethered at both ends and stretch the muscle and are then measuring the amount of phospholipase D (PLD) activity and the amount of phosphatidic acid production (the product created by PLD). Control = muscles that were not stretched, but went through the same procedure; Stretch = muscles that were stretched 15% for 15 minutes, 30 minutes, 60 minutes, or 90 minutes.

Primary Results:
- Direct measure of PLD activity increases in the first 15 minutes, but not afterward.
- Phosphatidic acid amount increases at all time point.

Take Away: The PLD activity data is unconvincing due to a lack of overall activity, but the phosphatidic acid content increase does lend evidence to stretch inducing phosphatidic acid increase.

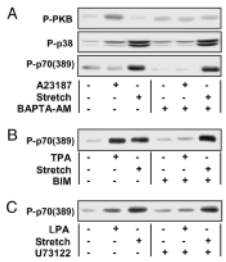

Fig. 4. Muscles were preincubated with media containing a pharmacological inhibitor for 30 min, subjected to mechanical stretch (Stretch +) or control condition (Stretch −), and stimulated with the appropriate pharmacological agonist for 90 min. The pharmacological inhibitors and agonists used, respectively, are BAPTA-AM (100 μM) and A23187 (20 μM) (*A*), BIM (5 μM) and TPA (1 μM) (*B*), and U73122 (10 μM) and lysophosphatidic acid (5 μM) (*C*). Appropriate solvent vehicles were used in all experiments. Samples were subjected to Western blot analysis of phosphorylated PKB (P-PKB) and p38 (P-p38) (*A*) or phosphorylated $p70^{S6k}$ [P-p70 (389)] (*A–C*). Blots are representative of at least three independent experiments.

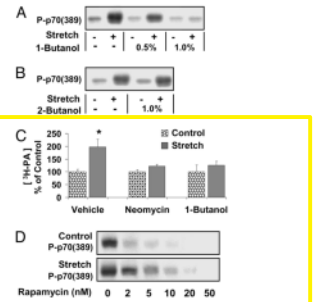

Fig. 5. A PLD-dependent increase in [PA] is necessary for mechanical activating of mTOR signaling. (*A* and *B*) Muscles were preincubated for 30 min with media containing the indicated concentration of 1-butanol (*A*) or 2-butanol (*B*), followed by an additional 90 min of mechanical stretch (Stretch +) or control condition (Stretch −). Samples were subjected to Western blot analysis for phosphorylated $p70^{S6k}$ [P-p70 (389)]. (*C*) Muscles were prelabeled with [^{3}H]arachidonate and then incubated for 30 min with media containing neomycin (10 mM), 1-butanol (1%), or the solvent vehicle, followed by an additional 90 min of mechanical stretch or control conditions, and the concentration of ^{3}H-labeled PA ([^{3}H-PA]) was determined. (*D*) Muscles were preincubated for 30 min with media containing 2–50 nM rapamycin, followed by an additional 90 min of stretch or control conditions. Samples were subjected to Western blot analysis for P-p70 (389). All blots are representative of at least three independent experiments. The graph represents the mean values ± SEM for each group expressed as a percentage of the treatment control (vehicle, neomycin, or 1-butanol), ($n = 3$–7 per group). *, significantly different from treatment control ($P \leq 0.05$).

involved in the mechanical activation of mTOR signaling and, therefore, suggest that the effect of neomycin was attributable to the inhibition of PLD.

1-Butanol Inhibits the Mechanical Activation of mTOR Signaling. To further assess whether the inhibition of PLD could prevent the mechanical activation of mTOR signaling, muscles were incubated with various concentrations of 1-butanol, a primary alcohol that inhibits PLD-catalyzed PA formation, or 2-butanol, a secondary alcohol that does not inhibit PLD-catalyzed PA formation (14, 24–26). These experiments demonstrate that 1-butanol produced a dose-dependent inhibition of the mechanically induced signaling to $p70^{S6k}$ (389), whereas 2-butanol had no effect (Fig. 5 *A* and *B*). To further confirm the specificity of 1-butanol, muscles were incubated with 1% 1-butanol and then stimulated with insulin; the results indicate that 1% 1-butanol did not inhibit insulin-induced signaling to PKB (presented in Fig. 7, which is published as supporting information on the PNAS web site). Because it has been reported that insulin-induced signaling to PKB is a PLD-independent signaling event (27), the lack of an effect of 1-butanol in this event is in concert with its specificity on PLD-dependent signaling. Taken together, these results indicate that mechanical stimuli use a PLD-dependent increase in [PA] to activate mTOR signaling.

Neomycin and 1-Butanol Inhibit the Mechanically Induced Increase in [PA]. To verify that PLD was required for the mechanically induced increase in [PA], muscles were incubated with PLD inhibitors (10 mM neomycin and 1% 1-butanol) and subjected to mechanical stimulation. The results indicated that both neomycin and 1-butanol blocked the mechanically induced increase in [PA] (Fig. 5*C*).

Mechanical Stimulation Confers Resistance to Rapamycin. PA has been reported to modulate mTOR signaling by binding to mTOR in its FRB domain (14). The FRB domain is also the binding site for the complex formed by the immunophilin FKBP12 and the drug rapamycin and is necessary for rapamycin to inhibit mTOR signaling (14). It has been proposed that the FKBP12–rapamycin complex and PA compete for binding to the FRB domain (14), and this hypothesis has been supported by the recent report that an elevation of PLD activity can confer resistance to rapamycin (28). To determine whether mechanical stimulation can also confer resistance to rapamycin, muscles were incubated with various concentrations of rapamycin, subjected to mechanical stimulation or control conditions, and analyzed for $p70^{S6k}$ (389) phosphorylation. The IC_{50} of rapamycin in mechanically stimulated muscles (2.32 ± 0.27 nM, $R^2 = 0.83$) was significantly higher than the IC_{50} in control muscles (1.06 ± 0.24 nM, $R^2 = 0.95$), ($P < 0.01$) (Fig. 5*D*). The higher IC_{50} of rapamycin in mechanically stimulated muscles demonstrates that mechanical stimulation confers resistance to rapamycin and suggests that the mechanical activation of mTOR signaling results from enhanced binding of PA to the FRB domain on mTOR.

Expression and Localization of PLD1, PLD2, and mTOR in Skeletal Muscle. To gain insight into how mechanical stimuli might regulate PLD activity, primary antibodies generated against the two isozymes of PLD (PLD1 and PLD2) and mTOR were used to determine the subcellular localization of these proteins in skeletal muscle. Analysis of different subcellular fractions of the EDL muscle indicated that the majority of PLD1 was localized

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Figure 5: The researchers are measuring phosphatidic acid levels (what phospholipase D produces) after no stretch (control) or stretching the muscle (stretch); then, they are measuring at without drug addition (vehicle) or with the addition of neomycin and 1-butanol (both are phospholipase D inhibitors) in 5C. In 5D, the researchers are measuring the amount of phospho-p70 in control muscle (no stretch) and stretched muscle (stretch) without rapamycin or with rapamycin at varying concentrations (50nM being the highest). This indicates the impact PLD has, through stretch, to produce phosphatidic acid and to stimulate phospho-p70, then how the inhibition of mTOR (the master growth stimulation molecule) by rapamycin affect phospho-p70 expression.

Primary Results:
- Inhibition of PLD reduces phosphatidic acid amount.
- Inhibiting mTOR reduces the amount of phosphorylated p70.

Take Away: Stretch induces phospho-p70 activity through mTOR, and likely through phosphatidic acid production, but this is not confirmed by this data.

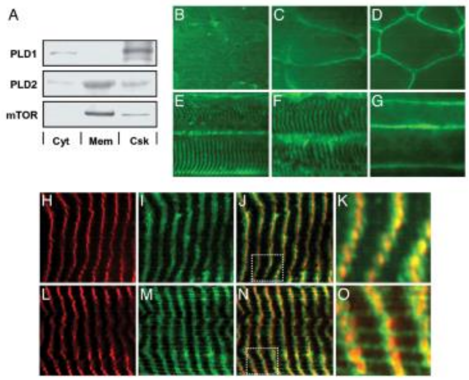

Fig. 6. Localization of PLD1, PLD2, and mTOR in skeletal muscle. (*A*) EDL muscles were separated into cytosol (Cyt), membrane (Mem), and cytoskeletal (Csk) fractions as described in *Supporting Methods*, and equal amounts of protein from each fraction were subjected to Western blot analysis for PLD1, PLD2, and mTOR. (*B–G*) Immunohistochemistry of cross-sections (*B–D*) and longitudinal sections (*E–G*) from the EDL muscle incubated with antibodies against PLD1 (*B* and *E*), PLD2 (*C* and *F*), or mTOR (*D* and G) and revealed with FITC-conjugated secondary antibodies by using an epifluorescent microscope. Longitudinal sections from the EDL muscle were also double-labeled with antibodies against α-actinin and PLD1 (*H–K*) or α-actinin and PLD2 (*L–O*). α-Actinin was revealed with TRITC-conjugated secondary antibodies (red). PLD1 and PLD2 were revealed with FITC-conjugated secondary antibodies (green). Images *H* and *I* were merged in *J*, which was magnified in *K*. Images in *L* and *M* were merged in *N*, which was magnified in *O*. Images in *H–O* were obtained by using a confocal microscope. All images are representative of at least three independent experiments. The boxes in *J* and *N* are the portions shown magnified in *K* and *O*.

to the cytoskeletal fraction, with only a small proportion of PLD1 in the cytosolic fraction. The majority of PLD2 was detected in the membrane and cytoskeletal fractions, with a small proportion in the cytosolic fraction. Similar to PLD2, mTOR was also localized to the membrane and cytoskeletal fractions (Fig. 6*A*). A quantitative analysis of these experiments is presented in Fig. 8, which is published as supporting information on the PNAS web site.

Immunohistochemical analysis of cross-sections of the EDL muscle demonstrated that PLD1 and PLD2 were distributed throughout the interior of the muscle fibers, but PLD2 also displayed a higher intensity of staining at the sarcolemmal membrane; mTOR was localized primarily at the sarcolemmal membrane (Fig. 6 *B–D*). Longitudinal sections of the EDL muscle provided additional evidence that mTOR is localized to the sarcolemmal membrane, whereas PLD1 and PLD2 revealed a distinct striated pattern (Fig. 6 *E–G*).

To further define the striated pattern that was observed for PLD1 and PLD2, double-labeling experiments were performed with antibodies directed against the z-band protein α-actinin. Confocal images from longitudinal sections demonstrate that both PLD1 and PLD2 are localized within and immediately adjacent to the region occupied by α-actinin. Furthermore, a pattern of thin fibrous structures running perpendicular to the z-band was observed for PLD2 but not PLD1 (Fig. 6 *H–O*). Previous studies have demonstrated by peptide competition that the PLD2 (29) and PLD1 (30) antibodies used in this study are highly specific.

Discussion

Previous reports have demonstrated that signaling by mTOR is required for mechanical-load-induced growth of skeletal muscle (3–5, 11). To date, the mechanisms involved in the mechanical activation of mTOR signaling have not been defined, but recent studies indicate that an unique (PI3K- and nutrient-independent) pathway is involved (2, 5, 13). This finding is surprising, because PI3K is generally considered to be indispensable for the activation of mTOR signaling (12, 31, 32). In fact, we have found only one other report of a PI3K-independent activation of mTOR signaling, where Wang and Proud (33) demonstrated that the Gq protein-coupled receptor agonist phenylephrine (PE) activated mTOR signaling through a wortmannin-insensitive (i.e., PI3K-independent) pathway. Later studies by Ballou *et al.* (34) implicated a role for PLD in this pathway, when it was shown that 1-butanol could inhibit the activation of mTOR signaling by PE. Therefore, this study was undertaken to test the hypothesis that mechanical stimuli activate mTOR signaling through a PLD-dependent mechanism.

The results from this study provide several lines of evidence to support the hypothesis that mechanical stimuli activate mTOR signaling through a PLD-dependent increase in [PA]. First, an elevation in [PA] was sufficient for the activation of mTOR signaling (Fig. 1). Second, the isozymes of PLD (PLD1 and PLD2) are localized to the z-band in skeletal muscle (a critical site of mechanical force transmission) (Fig. 6). Third, mechanical stimulation induced PLD activation, PA accumulation, and mTOR signaling (Figs. 2–5). Fourth, pharmacological inhibition of PLD blocked the mechanically induced increase in

[PA] and activation of mTOR signaling (Figs. 3 and 5). Finally, mTOR signaling was partially resistant to rapamycin in mechanically stimulated muscles (Fig. 5), suggesting that the mechanical activation of mTOR signaling resulted from enhanced binding of PA to the FRB domain on mTOR.

Although our results indicate that PLD is the enzyme responsible for the mechanically induced increase in [PA], we cannot rule out the potential contribution of additional PA regulatory enzymes. Such enzymes might include the PA phosphatases (PAP), which dephosphorylate PA to DAG, and the DAG kinases (DAGK), which generate PA through the phosphorylation of DAG (35, 36). In fact, previous studies have shown that PLD-derived PA can be rapidly converted to DAG by PAP and that DAG can, in turn, be transformed back to PA by DAGK (37). Such a mechanism might explain why mechanical stimulation produced a transient increase in PLD activity, whereas the elevation in [PA] was sustained. Additional studies will be needed to fully define the mechanisms by which PLD contributes to the mechanically induced increase in [PA].

Our finding that mechanical stimulation promoted an increase in PLD activity raises questions about the mechanism(s) involved in this process. Previous studies have shown that PLD activity can be regulated by various protein kinases such as PKC, protein-tyrosine kinases, and the MAP kinase family as well as the small G proteins from the ARF, Rho, and Ras families (38, 39). Furthermore, the activity of PLD has been shown to be regulated through interactions with various cytoskeletal proteins such as tubulin, actin, and α-actinin (40–42). For example, Park *et al.* (41) demonstrated that PLD can bind to α-actinin and that this interaction inhibits the activity of PLD. This observation is of particular interest, because our results demonstrated that both isozymes of PLD are colocalized with α-actinin in skeletal muscle. Because α-actinin is a vital component of the z-band in skeletal muscle, and the z-band is a focal point for mechanical force transmission (43), it is tempting to speculate that mechanical stimuli induce a physical dissociation of PLD from α-actinin and relieve PLD from the inhibitory effect of the interaction. Additional interest in the cytoskeletal network comes from the observation that disruption of the actin cytoskeleton with cytochalasin D can prevent the mechanical activation of signaling to $p70^{S6k}$ in skeletal muscle cells *in vitro* (44). Clearly, the mechanisms responsible for the mechanical activation of PLD activity remain to be more fully defined, and the role of the cytoskeletal network in this process will be an area worthy of further investigations.

In summary, the results of this study indicate that mechanical stimuli activate mTOR signaling through a PLD-dependent increase in [PA]. Because the activation of mTOR signaling is required for mechanically induced growth of skeletal muscle, these findings contribute significantly to our understanding of how mechanical signals are transduced into the molecular events that regulate skeletal muscle growth and remodeling.

Materials and Methods

Materials. Details regarding the specific materials used in this study are described in *Supporting Methods*, which is published as supporting information on the PNAS website.

Animal Care and Use. All experimental procedures were approved by the University of California at San Diego Animal Care and Use Committee. Male C57BL6 mice (The Jackson Laboratories and Harlan Laboratories), 8–14 weeks of age, were randomly assigned to different experimental groups. All animals were allowed free access to food and water.

Organ Culture and Mechanical Stimulation. Mice were anesthetized with sodium pentobarbital (150 mg/kg of body weight), and the EDL muscle of the hind limb was placed in the *ex vivo* organ culture system at optimal length (Lo) as described in ref. 13. Details regarding the protocol for mechanical stimulation have been described in ref. 5. Briefly, 15% intermittent passive stretch was used as a source of mechanical stimulation; in addition, muscles were maintained at Lo as a control condition.

Pharmacological Inhibitors. Muscles were preincubated with pharmacological inhibitors or the solvent vehicle for 30 min before stimulation with pharmacological agonists or mechanical stretch. All pharmacological inhibitors were present throughout the entire stimulation period. Stock solutions of the inhibitors BAPTA-AM, BIM, U73122, and rapamycin were dissolved in DMSO, neomycin was dissolved in distilled (d)H_2O, and 1-butanol and 2-butanol were added directly to the culture media.

Pharmacological Agonists. Muscles were incubated with the pharmacological agonists or solvent vehicles for 90–120 min as indicated in the figure legends. Stock solutions of the pharmacological agonists TPA and A23187 were dissolved in DMSO and lysophosphatidic acid was dissolved in PBS with 0.1% (wt/vol) BSA. Insulin and propranolol were dissolved in dH_2O.

Western Blot Analysis and Sample Preparation. Unless otherwise noted, muscles were removed from the organ culture system, immediately frozen in liquid nitrogen, homogenized, and subjected to Western blot analysis as described in ref. 13. Details regarding the procedure for separating the cytosolic, membrane, and cytoskeletal muscle fractions are provided in *Supporting Methods*.

Immunohistochemistry. EDL muscles were mounted at resting length in optimal cutting temperature compound and frozen in isopentane chilled with liquid nitrogen. Longitudinal and cross-sections (10 μm) were generated with a cryostat and immediately fixed in −20°C acetone or −20°C methanol. Fixed sections were incubated with PLD1, PLD2, mTOR, or α-actinin primary antibodies. Primary antibodies were detected with FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies and visualized with an epifluorescent or confocal microscope. Additional details regarding these procedures are provided in *Supporting Methods*.

Analysis of PLD Activity and [PA]. PLD activity was measured with the transphosphatidylation assay described by Facchinetti *et al.* (24). Briefly, muscles were prelabeled in the organ culture system with media containing [^{3}H]arachidonate (1 μCi/ml) (1 Ci = 37 GBq) for 2 h and then subjected to experimental treatments. PLD activity was measured during the final 15 min before the indicated treatment time by washing the muscle with fresh media for 5 min and then incubating with media containing 0.5% 1-butanol for 15 min. The same procedure was used for measurements of ^{3}H-labeled PA, except 1-butanol was not added to the culture media. Samples were homogenized in chloroform–methanol 2:1 (vol/vol) with a polytron, and lipids were extracted according to Folch *et al.* (45). Phosphotidylbutanol (PtdBut) and PA standards (10 μg) were added to the extracted lipids, and aliquots were used for the measurement of radioactivity in the total lipids or spotted on LK5D silica gel plates for separation of PtdBut and PA by TLC. The plates were developed with a solvent system consisting of ethyl acetate–isooctane–acetic acid–water 13:2:3:10 (vol/vol). This system provided efficient separation of PtdBut (R_f = 0.41) and PA (R_f = 0.14), which were visualized by iodine staining. Iodine-stained spots containing the ^{3}H-labeled PtdBut and PA were scraped off the TLC plate, and the radioactivity was measured by liquid scintillation spectrometry. Final calculations for PLD activity and [PA] were made by

1. This is potential explanation for figures 2A&B.

dividing the amount of radioactivity in the PtdBut or PA spot by the amount of radioactivity in the total lipid extract.

Statistical Analysis. All values are expressed as means ± SEM. Statistical significance was determined by using ANOVA, followed by Student–Newman–Keuls post hoc analysis. Differences between groups were considered significant if $P \leq 0.05$.

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