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Insulin, Insulin-like Growth Factor-I, and Platelet-Derived Growth Factor Activate Extracellular Signal-Regulated Kinase by Distinct Pathways in Muscle Cells

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We have investigated the signaling pathways initiated by insulin, insulin-like growth factor-1 (IGF-I), and platelet-derived growth factor (PDGF) leading to activation of the extracellular signal-regulated kinase (ERK) in L6 myotubes. Insulin but not IGF-I or PDGF-induced ERK activation was abrogated by Ras inhibition, either by treatment with the farnesyl transferase inhibitor FTP III, or by actin disassembly by cytochalasin D, previously shown to inhibit Ras activation. The protein kinase C (PKC) inhibitor bisindolylmaleimide abolished PDGF but not IGF-I or insulin-induced ERK activation. ERK activation by insulin, IGF-I, or PDGF was unaffected by the phosphatidylinositol 3-kinase inhibitor wortmannin but was abolished by the MEK inhibitor PD98059. In contrast, activation of the pathway involving phosphatidylinositol 3-kinase (PI3k), protein kinase B, and glycogen synthase kinase 3 (GSK3) was mediated similarly by all three receptors, through a PI 3-kinase-dependent but Ras- and actin-independent pathway. We conclude that ERK activation is mediated by distinct pathways including: (i) a cytoskeleton- and Ras-dependent, PKC-independent, pathway utilized by insulin, (ii) a PKC-dependent, cytoskeleton- and Ras-independent pathway used by PDGF, and (iii) a cytoskeleton-, Ras-, and PKC-independent pathway utilized by IGF-I.

Key Words: **mitogen-activated protein kinase; MAPK; extracellular signal regulated kinase; ERK; actin cytoskeleton; protein kinase B; PKB; Akt; glycogen synthase kinase 3; GSK3.**

Receptor tyrosine kinases such as those for insulin, insulin-like growth factor-I (IGF-I), and platelet-derived growth factor (PDGF) stimulate nuclear events by activation of cascades of protein kinases (1). The cytoplasmic portions of the receptors or associated adapter molecules such the insulin receptor substrates (IRS) and the Src and collagen homologues (Shc) become phosphorylated on tyrosine residues upon hormone binding. This serves to attract other signaling molecules such as the adapter Grb2, that are coupled to effector molecules such as the guanine nucleotide exchange factor Sos (1–3) and phosphatidylinositol 3-kinase (PI 3-kinase) (4, 5).

The activated Shc-Grb2-Sos complex activates the small GTP-binding protein Ras, which initiates a cascade of serine/threonine kinases, some of which eventually translocate to the nucleus to stimulate gene expression (6, 7). The extracellular signal regulated MAP kinases, ERK 1 and 2, are key intermediates in the propagation of signals from many growth factor receptors to nuclear events (6, 8). ERKs are activated via Ras, Raf-1 and MEK, a dual function kinase that phosphorylates ERK on tyrosine and threonine residues leading to ERK activation (9, 10). Since all three growth factors, insulin, IGF-I and PDGF, activate both Ras and ERK (11), it has generally been assumed that all three utilize this conserved pathway to mediate ERK activation and gene expression.

IRS lies upstream of a signaling cascade involving PI 3-kinase. When phosphorylated by this lipid kinase, 3′-phosphoinositides recruit phosphoinositide-dependent kinases such as phosphoinositide-dependent kinase (PDK) to plasma membrane. This triggers a separate cascade of protein kinases such as protein kinase B (PKB, also known as Akt) and the glycogen synthase kinase 3 (GSK3). When activated by PDK, Akt phosphorylates and inhibits GSK3. The Akt/GSK3 pathway

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is essential in the regulation of both metabolic and nuclear events such as glycogen synthesis and gene expression and apoptosis, respectively (12–14).

We have previously reported that an intact actin cytoskeleton is required for the propagation of the insulin induced signaling cascade downstream of Shc, involving Grb2/Ras/ERK, leading to the expression of the transcription factor c-*fos* and DNA synthesis (15). Our work suggested that the actin cytoskeleton facilitates the propagation of the Ras-ERK pathway by mediating the interaction of the two adaptors, Shc and Grb2, an event that is essential for Ras activation and downstream signaling (15). While recent studies have suggested an interaction of growth factor receptors with the integrin signaling pathways (16), the role of the actin cytoskeleton in the transmission of intracellular signals initiated by IGF-I and PDGF is not known.

In the present study, we sought to identify the mechanisms by which the PDGF and IGF-I receptors mediate ERK activation in muscle cells and to compare and contrast them with the signaling pathways used by insulin. Our results indicate that insulin, IGF-I and PDGF utilize at least three separate pathways leading to ERK activation, and emphasize that this pivotal kinase is situated at the convergence of signaling pathways leading to nuclear events.

EXPERIMENTAL PROCEDURES

Materials. α-Minimum essential medium (α-MEM) and fetal bovine serum, antibiotic/antimycotic were from GIBCO/BRL (Burlington, ON). Human insulin was obtained from Eli Lilly Co. (Toronto, ON), insulin-like growth factor-I (IGF-I) was from KabiPharmacia Peptide Hormones (Stockholm, Sweden), and platelet-derived growth factor (PDGF) was purchased from UBI (Lake Placid, NY). Cytochalasin D and all chemicals for SDS–PAGE and immunoblotting were obtained from Sigma Chemicals Co. (St. Louis, MO). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Buckinghamshire, England). The Mek inhibitor PD98059, bisindolylmaleimide (BIM) and the Ras farnesyl transferase inhibitor FTP III were obtained by CalBiochem (La Jolla, CA). The monoclonal anti-Phospho-ERK (anti-P-ERK), anti-Phospho-AktSer473 (anti-P-Akt), and anti-Phospho-GSK3α/β (anti-P-GSK3) antibodies were obtained from New England Biolabs (Beverly, MA) while the monoclonal anti-Ras antibody from Quality Biotech (Camden, NJ).

Cell culture, incubations, and treatments. Monolayers of L6 muscle cells were grown to the stages of myotubes (day 7) as previously described (17). The cells were grown in 12-well plates (diameter of well 2.5 cm) for whole cell lysate preparations or in 10-cm-diameter dishes for precipitations and Ras activity experiments. Cells were serum-deprived for at least 5 h prior to experimental manipulations. For Ras inhibition, cells were treated with 10 μM FTP III for 24 h. Cell treatments with cytochalasin D (1 μM), wortmannin (100 nM), and Mek inhibitor PD98059 (50 μM) were for 1 h prior to a 5 min incubation period with insulin (100 nM), IGF-I (3 nM), and PDGF (30 nM). At the end of all treatments, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were either lysed for whole cell lysate preparations or Ras precipitation as described below.

Immunoblotting of whole cell lysates. Following the indicated treatments, cells were lysed with 4× Laemmli sample buffer. Fifty micrograms of cell lysates were subjected to SDS–PAGE and immunoblotted with anti-P-ERK, anti-P-Akt(Ser473), and anti-P-GSK3α/β specific antibodies as previously described (18).

GTP-Ras precipitation. Ras activation was estimated by evaluation of GTP-bound Ras levels purified by precipitation from treated and control myotubes. A GST-fusion protein of the Ras-binding domain of c-Raf (RBD-Raf) was amplified in *E. coli* and used for GTP-Ras precipitation according to the method by Taylor and Shalloway (1996) (19).

Quantitation. The immunoblotting results of all experiments were scanned and quantitated using ImageQuant softwear from Molecular Dynamics (Sunnyvale, CA). The effects of growth factors alone were expressed as fold change relative to control basal, while the effects of the inhibitors on growth factor stimulation were expressed as percent (%) change relative to stimulated control. Statistical analysis was done by analysis of variance (ANOVA) with correction for multiple comparison (Sheffe). Significance was considered to be a P value of <0.05.

RESULTS

Effect of Actin Disassembly and Ras Inhibition on ERK Activation

Our previous studies in L6 muscle cells indicated that an intact actin cytoskeleton is required for propagation of signals along the Shc-Ras-ERK pathway because it facilitates Shc interaction with Grb2 and activation of Ras (15). Therefore, we first examined whether IGF-I and PDGF also utilize the actin-dependent Shc-Grb2/Sos-Ras-Raf-MEK pathway to mediate ERK activation. Figure 1 shows representative immunoblots from five independent experiments. In serum-deprived myotubes in the absence of growth factors, there was minimal phosphorylation of ERK. All three growth factors induced a robust increase in ERK phosphorylation (average increases for insulin: 3600-fold, IGF-I: 5700-fold, and PDGF: 6400-fold), as detected by immunoblotting with an anti-phospho-ERK specific antibody (Fig. 1, top panel). These studies also revealed that both isoforms of ERK, ERK 1 and 2, were phosphorylated appearing as a double band in the 42–44 kDa range. This dual phosphorylation has been shown by us and others to correlate with an increase in kinase activity of the enzyme (15, 20). Consistent with our earlier observations (15), we observed (Fig. 1, top panel) that actin disassembly significantly inhibited insulin-induced activation of ERK (average 80.27 $\pm$ 7.36% inhibition). Furthermore, ERK activation by insulin was significantly inhibited by pretreatment of the cells with the Ras inhibitor (FTP III; Fig. 1, bottom panel) (average 90.05 $\pm$ 2.98% inhibition). By contrast, the IGF-I- and PDGF-induced phosphorylation of ERK was not significantly affected by either actin disassembly or Ras inhibition (Fig. 1). Actin disassembly caused a small, but statistically insignificant inhibition of IGF-I-induced ERK phosphorylation (average 12.03 $\pm$ 3.6%) but did not inhibit and in fact increased the phosphorylation of this enzyme by PDGF (an average 18.55 $\pm$ 8.4% increase, NS). Inhibition of Ras with FTP

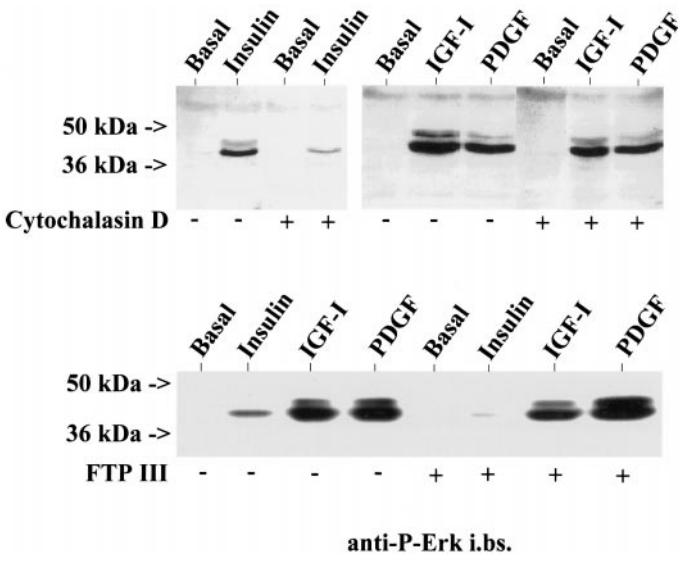

FIG. 1. Effects of actin disassembly and Ras inhibition on the insulin, IGF-I, and PDGF-induced ERK phosphorylation. Serum-deprived myotubes were preincubated with either 10 μM FTP III for 24 h or 1 μM cytochalasin D for 1 h or were left untreated before a 5-min stimulation of cells with either insulin (100 nM), IGF-I (3 nM), or PDGF (30 nM). Following these treatments the cells were rapidly washed in ice-cold buffer, lysed in Laemli sample buffer, and subsequently subjected to SDS–PAGE and immunoblotting (i.b.) with an anti-phospho-ERK specific antibody as described under Experimental Procedures.

III did not prevent the phosphorylation and activation of ERK by either IGF-I or PDGF. These observations connote that IGF-I and PDGF: (i) utilize mechanisms distinct from that of insulin to mediate ERK activation, and (ii) can activate ERK and presumably downstream nuclear events in a Ras-independent fashion. Ras-independent activation of ERK has been reported previously in response to integrin activation in mouse NIH 3T3 fibroblasts (21) and in response to angiotensin II stimulation of vascular smooth muscle cells (22). However, it is noteworthy that in both of these cell types, PDGF-induced ERK activation was Ras-dependent (21, 22), reflecting perhaps differences in signal transduction between different cell types.

Effect of Actin Disassembly on Ras Activation

The next experiments were designed to assay directly the status of Ras activation under the conditions of our experiments using the Ras-binding domain of Raf to purify active Ras from protein extracts followed by immunoblotting the purified complexes with anti Ras antibody. Figure 2 shows representative immunoblots from 3 independent experiments. Using this assay, we observed that quiescent, serum-deprived cells had minimal levels of active Ras and that insulin treatment induced robust activation of Ras (4700-fold increase). Importantly, the Ras farnesylation inhibitor, FTP III, completely abolished insulin-induced Ras activation (average 94 $\pm$ 5.3% inhibition) (Fig. 2, top panel). Similar to insulin, treatment of myotubes with IGF-I or PDGF induced vigorous activation of Ras (6100-fold and 5200-fold, respectively). Actin disassembly abolished Ras activation in response to insulin, IGF-I, and PDGF (98 $\pm$ 2.3%, 99 $\pm$ 5.6%, and 86 $\pm$ 6.3% inhibition, respectively). These data confirmed our previous observations that Ras activation by insulin is dependent on an intact actin cytoskeleton that facilitates interaction of Shc and Grb2 (15), and suggest a similar mechanism of Ras activation for the growth factors IGF-I and PDGF.

Role of Mek and Phosphatidylinositol 3-Kinase in ERK Activation

Previous studies suggested that prolonged ERK activation can be achieved independently of MEK (23), while other reports indicated a role of phosphatidylinositol 3-kinase (PI 3-kinase) in ERK activation by regulating signaling events at the level of Ras (24) or PKC (22). We investigated the role of MEK and PI 3-kinase in ERK activation using specific inhibitors of the two enzymes. Figure 3 shows a representative immunoblot from four independent experiments. Pre-incubation of myotubes with the PI 3-kinase inhibitor wortmannin, at concentrations known to abolish PI 3-kinase activity (25, 26), left unaffected the ERK phosphorylation by insulin, IGF-I or PDGF (Fig. 3). By contrast pre-

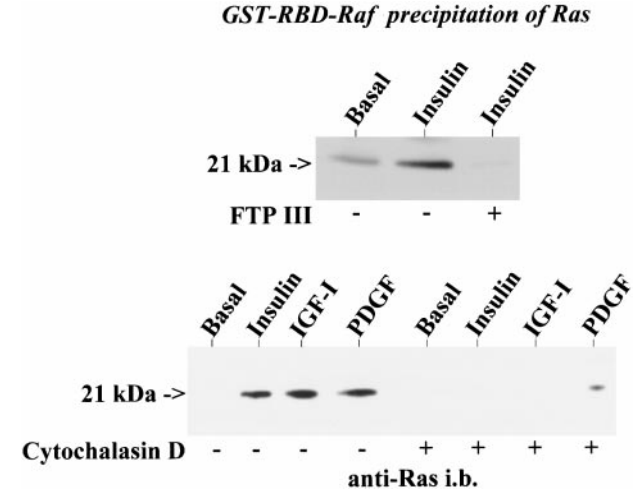

FIG. 2. Actin disassembly and FTP III inhibit Ras activation by insulin, IGF-I, and PDGF. Serum-deprived myotubes grown in 10-cm-diameter dishes were preincubated with either 10 μM FTP III for 24 h (top panel), 1 μM cytochalasin D for 1 h (bottom panel), or left untreated. Then the cells were stimulated with either insulin (100 nM), IGF-I (3 nM), or PDGF (30 nM) for 2.5 min. Following these treatments the cells were rapidly washed, lysed on ice, and GTP-Ras was precipitated from lysates as described under Experimental Procedures. Precipitates were then examined by immunoblotting (i.b.) using a specific anti-Ras antibody.

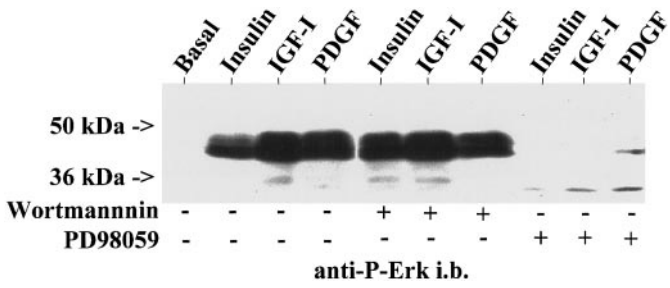

FIG. 3. Effects of phosphatidylinositol 3-kinase and MEK inhibition on ERK phosphorylation by insulin, IGF-I, and PDGF. Serum-deprived myotubes were incubated with either 100 nM wortmannin, 50 μM PD98059, or left untreated for 1 h followed by stimulation of the cells with insulin (100 nM), IGF-I (3 nM), or PDGF (30 nM) for 5 min. Following these treatments the cells were washed and lysed in sample buffer and analyzed by immunoblotting (i.b.) using an anti-phospho-ERK antibody.

incubation of myotubes with the MEK inhibitor PD98059 abolished ERK activation by all three stimuli (average inhibitions of 95 ± 3.6%, 99 ± 7.2%, and 91 ± 8.7% for insulin, IGF-I and PDGF, respectively). Taken together, these observations indicate that all three tyrosine kinase receptors activate ERK by a MEK-dependent but PI 3-kinase-independent pathway.

Role of Protein Kinase C in ERK Activation

Previous studies have indicated that conventional isoforms of protein kinase C may be involved in Ras activation. Phorbol ester treatment of cells, which activates both conventional and novel, diacylglycerol-sensitive, PKC isoforms induces ERK activation (27, 28). Other studies using specific PKC inhibitors have also suggested that activation of MAP kinases involves PKC (29–32). We therefore investigated the involvement of members of the PKC family in the ERK activation by insulin, IGF-I, and PDGF using the specific PKC inhibitor bisindolylmaleimide (BIM) (33). BIM was used at the concentration of 1 μM which is expected to inhibit most PKC isoforms except the atypical ones such as PKC_ζ and PKC_λ, which are inhibited only by higher concentrations of BIM (>10 μM) (33, 34). Figure 4 shows a representative immunoblot from four independent experiments. Preincubation of cells with BIM inhibited the PDGF- (average inhibition 99.2 ± 4.7%) but not insulin or IGF-I-induced ERK activation indicating a specific involvement of PKC in the PDGF signaling pathway upstream of ERK. Neither higher concentrations of BIM (5 and 10 μM), nor specific inhibitors of PKC_ζ (a cell-permeable, myristoylated PKC_ζ pseudo-substrate) and $PKC_{\beta II}$ (LY379196) (35) inhibited insulin or IGF-I-induced phosphorylation of ERK (results not shown). Additionally, the specific PKC_ζ and $PKC_{\beta II}$ inhibitors did to inhibit PDGF-induced ERK phosphorylation (results not shown). These observations suggest involvement of conventional (other than $PKC_{\beta II}$) and/or of novel PKC isoforms in PDGF-induced ERK activation.

Mechanism of Akt Activation and GSK3 Phosphorylation by Insulin, IGF-I, and PDGF

We next investigated the mechanism by which insulin, IGF-I, and PDGF mediate activation of Akt and GSK3 using immunoblotting of whole cell lysates with phosphorylation-specific antibodies. Figure 5 shows a representative immunoblot from 4 independent experiments. All three growth factors, insulin, IGF-I, and PDGF induced comparable levels of phosphorylation of both Akt and GSK3, the latter indicative of Akt activation (Fig. 5). Neither actin disassembly nor Ras inhibition with FTP III prevented phosphorylation of either Akt or GSK3 suggesting that these kinases are regulated by signaling pathways that are independent of the Shc-Ras-MAPK pathway. By contrast, inhibition of PI 3-kinase by wortmannin dramatically inhibited Akt phosphorylation by insulin (81 ± 5.4%), IGF-I (92 ± 6.2%), and PDGF (91 ± 7.3%) and abolished the ability of this kinase to phosphorylate GSK3 in vivo (95 ± 6.8%, 98 ± 7.5%, and 99 ± 5.6% inhibition for insulin, IGF-I, and PDGF, respectively, see Fig. 5). These results indicate that, unlike ERK, all three growth factors utilize the same PI 3-kinase-dependent pathway to activate Akt and GSK3 in myotubes.

DISCUSSION

In this article we have provided evidence that the transmembrane tyrosine kinase receptors for insulin, IGF-I, and PDGF utilize both common and distinct pathways to mediate ERK activation in muscle cells. By contrast, activation of Akt and GSK3 by all three receptors proceeds by a conserved, PI 3-kinase-dependent signaling pathway.

With respect to ERK activation in muscle cells, it is likely that at least three different pathways exist

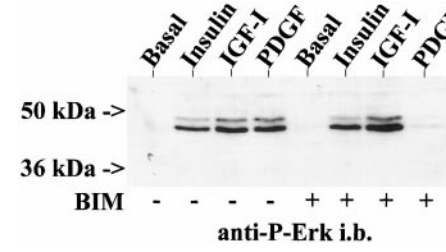

FIG. 4. Protein kinase C inhibition inhibits PDGF-induced but not insulin- or IGF-I-induced ERK phosphorylation. Serum-deprived myotubes were either incubated with 1 μM bisindolylmaleimide (BIM) or left untreated for 1 h followed by stimulation of the cells with insulin (100 nM), IGF-I (3 nM), or PDGF (30 nM) for 5 min. Following these treatment the cells were washed and lysed in sample buffer and analyzed by immunoblotting (i.b.) using an anti-phospho-ERK antibody.

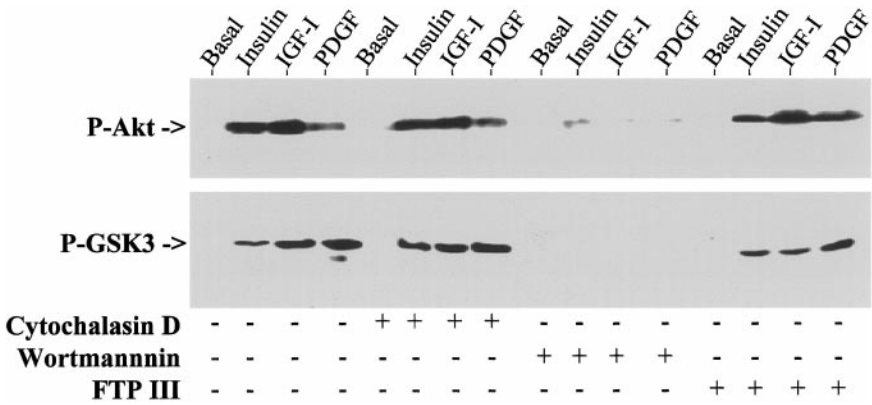

FIG. 5. Mechanism of Akt and glycogen synthase kinase 3 (GSK3) regulation by insulin, IGF-I, and PDGF. Serum-deprived myotubes were incubated with either 10 μM FTP III for 24 h or with 1 μM cytochalasin D (BIM), 100 nM wortmannin, or left untreated. Next the cells were stimulated with insulin (100 nM), IGF-I (3 nM), or PDGF (30 nM) for 5 min. Following these treatment the cells were washed and lysed in sample buffer and analyzed by immunoblotting (i.b.) using specific anti-phospho-Akt(Ser473) and anti-phospho-GSK3 antibodies.

which converge on the ERK kinase, MEK. Direct inhibition of Ras with FTP III or indirect inhibition through actin disassembly with cytochalasin D, demonstrated that insulin signals through this small G protein to activate ERK. On the other hand, IGF-I and PDGF appear to utilize primarily Ras-independent pathways to activate ERK in myotubes. The concept of Ras-independent activation of ERK has been proposed earlier (36–39) but has not been demonstrated previously for either IGF-I or PDGF, which have generally been reported to require Ras activation for initiation of the Mek-ERK pathway (40, 41).

IGF-I appears to activate ERK by a pathway distinct from PDGF. Our observations indicate a role of PKC in PDGF-induced ERK activation compatible with previous studies in other cell types (42–44). Although our data do not allow us to identify unequivocally the PKC isoform involved in PDGF activation of ERK, the concentration of BIM required for complete inhibition of PDGF action (1 μM), suggests that one of the conventional and/or the novel PKC isoforms may be involved. Previous studies (45, 46) have demonstrated the presence of PKC α, β_{I} and β_{II}, δ, ϵ, and ζ in L6 myotubes. Therefore, it is possible that PKCα, β_{I}, δ, or ϵ is involved in PDGF-induced activation of ERK since both the $\mathrm{PKC}_{\beta\mathrm{II}}$ and PKC_{ζ} inhibitors were ineffective at blocking ERK activation. In summary unlike insulin and IGF-I, PDGF utilizes a cytoskeleton- and Ras-independent but PKC-dependent pathway to mediate ERK activation in myotubes. Additional studies will be required to elucidate this pathway.

The demonstration that insulin and IGF-I utilize different mechanisms to activate ERK in muscle cells was unexpected. The striking structural similarities between insulin and IGF-I as well as their respective receptors, combined with earlier studies on the mechanisms of IGF-I signal transduction (47) suggested that the two receptors would share similar signaling pathways. The results of the current study have demonstrated that IGF-I may be able to activate ERK by a novel pathway, that although involving Mek, may be independent of Ras-, PKC-, and PI 3-kinase. Moreover, this pathway does not depend on an intact cytoskeleton. Our observations are also compatible with the involvement of multiple signaling pathways in the IGF-I induced ERK activation. In muscle cells, unlike other cell types, the IGF-I receptor may be able to simultaneously activate Ras-dependent and independent pathways to induce ERK activation. In this context, independent inhibition of these pathways may not suffice to abolish the ability of the receptor to transmit signals to ERK. Our future studies will focus on the specific mechanisms of action of IGF-I in muscle cells to identify the participants of its signaling pathway.

The present studies provide evidence supporting the concept that the actin cytoskeleton is selectively involved in growth factor signaling. Our earlier work (15) demonstrated that an intact cellular actin network is required for the successful propagation of insulin action by facilitation of the interaction of Shc with Grb2. We interpret these observations to mean that the activation of Ras is the key cytoskeleton-dependent event that is required for insulin action. Although IGF-I and PDGF also activate Ras in a cytoskeleton-dependent manner (see Fig. 2), alternate pathways are activated by these growth factors that lead to ERK activation. These alternate (cytoskeleton-independent) pathways may be involved in IGF-I and PDGF stimulation of nuclear events in cells with less stable cytoskeleton such as cancer cells with malignant metastatic potential (48, 49). The existence of distinct pathways leading to ERK activation distinguishes the effects of PDGF and IGF-I from that of insulin and may account for the observation that these two growth factors function as stronger growth promoters as compared to insulin (50, 51). It is therefore apparent that understanding the

molecular details of the signaling cascades utilized by these receptors may facilitate the development of therapeutic strategies to inhibit uncontrolled proliferation of neoplastic cells.

In summary, the present study shows that in muscle cells, (i) insulin requires an intact cytoskeleton and utilizes Ras to mediate ERK activation, (ii) PDGF-induced ERK activation involves BIM-sensitive PKC isoforms, and (iii) IGF-I-mediated ERK activation may be mediated by a novel pathway or multiple pathways. Future studies are needed to identify the signaling intermediates involved in IGF-I- and PDGF-induced ERK activation and downstream nuclear events.

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