Synergistic Effects of Insulin and Phorbol Ester on Mitogen-activated Protein Kinase in Rat-1 HIR Cells*

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Regulation of the activity of the extracellular signal regulated kinase (ERK) mitogen-activated protein kinases was examined in Rat-1 HIR, a fibroblast cell line overexpressing the human insulin receptor. Insulin or phorbol ester induced partial activations of ERKs, while a combination of insulin and phorbol ester resulted in a synergistic activation. Preincubation with phorbol ester increased the subsequent response to insulin. Phorbol ester did not enhance tyrosine phosphorylation of the insulin receptor. Insulin did not enhance activation of phospholipase D in response to phorbol ester. Lysophosphatidic acid also acted synergistically with insulin to induce ERK activation. Lysophosphatidic acid alone had little effect on ERK, and did not activate phospholipase D. The combination of phorbol ester and insulin maintained tyrosine phosphorylation of focal adhesion kinase, while insulin alone decreased its tyrosine phosphorylation. Phorbol ester induced phosphorylation of Shc on serine/threonine, while insulin induced tyrosine phosphorylation of Shc and Shc-Grb2 binding. These results suggest that full activation of ERKs in fibroblasts can require the cooperation of at least two signaling pathways, one of which may result from a protein kinase C-dependent phosphorylation of effectors regulating ERK activation. In this manner, phorbol esters may enhance mitogenic signals initiated by growth factor receptors.

The ERK mitogen-activated protein kinases (MAPks), which are activated in response to a wide variety of growth factors, hormones, and other mitogenic stimuli, have been linked to the induction of cell proliferation (1). The insulin receptor, a tyrosine kinase, can elicit both metabolic and mitogenic responses (2). The insulin receptor binds to several effectors, with binding to insulin receptor substrate-1 (IRS-1) mediating most of the metabolic effects of insulin. Mutation or deletion of some of the phosphorylatable tyrosines in the insulin receptor can enhance its mitogenic activity (3), suggesting that multifunctionality of the receptor may compromise its mitogenic potential. Phorbol ester tumor promoters can act additively or synergistically with insulin to induce mitogenesis (4, 5).

The mitogenic activity of the insulin receptor has been correlated with its ability to activate MAPK, a response that is mediated predominantly through interaction of the insulin receptor with Shc (6). IRS-1 and Shc compete for binding to Grb2, such that IRS-1 may either inhibit (7) or enhance (8) activation of MAPK. Tyrosine-phosphorylated Shc binds to the adapter Grb2, causing disassembly of SOS from Grb2 resulting in GDP-GTP exchange on Ras (9). GTP-bound Ras promotes activation of Raf-1 kinase (1, 10). Raf-1 phosphorylates and activates MEK (mitogen-activated protein kinase kinase) via PKC, which phosphorylates and activates MAPK. One downstream effector of MAPK is RSK, another protein serine/threonine kinase. It is clear that the insulin receptor initiates signals that are independent of MAPK activation (reviewed in Ref. 11).

Activation of Ras is not required for metabolic signaling by insulin (12), and activation of MAPK is not sufficient for these metabolic responses (13, 14).

Focal adhesion kinase (FAK), a tyrosine kinase localized to focal adhesions, is involved in the association of cells with the extracellular matrix via integrins. While FAK is constitutively phosphorylated on tyrosine in quiescent fibroblasts (15), its phosphorylation is increased in response to phorbol esters (16).

Phosphorylation of FAK enhances its binding to Grb2 (17) and phosphatidylinositol 3-kinase (18), and results in activation of the MAPK cascade (17). Insulin induces dephosphorylation of FAK in Rat-1 HIR cells (15). Similar results have been reported for another cell line (19).

Phorbol esters, which activate isoforms of protein kinase C (PKC), are general activators of the MAPK cascade. The pathway involved in this response is not clear. Activation of MAPK via PKC appears to involve the Ras/Raf-1 pathway in some cases (20, 21), but not in others (22). PKC can phosphorylate the insulin receptor on serine and threonine residues, including serine 1327 (23). These phosphorylations can decrease the tyrosine kinase activity of the receptor (24) and may have additional effects on its substrate recognition (23).

Activation of PKCα interferes with phosphorylation of IRS-1 by the insulin receptor (24). PKC activation results in enhanced association of Shc with a cytosolic protein tyrosine phosphatase, PTP-PEST (25). Thus, PKCs can potentially affect insulin receptor signaling at several levels.

In this study, we have examined the activation of MAPK in Rat-1 fibroblasts expressing the human insulin receptor. A strikingly synergistic activation of MAPK was observed in confluent cells incubated with both insulin and phorbol ester. The mechanism of the synergism appears to reflect a requirement...
for activation of two signaling pathways for full induction of MAPK activity.

MATERIALS AND METHODS

Cell Culture—Rat-1 HIR cells are rat embryo fibroblasts stably expressing the human insulin receptor (HIR) under the control of the SV40 early promoter (26). These were originally provided by Dr. D. McClain (University of Mississippi), and had been maintained in our laboratories for more than 2 years. The cells were maintained in a Dulbecco’s modified Eagle’s (DME) medium supplemented with 10% fetal calf serum (Upstate Biotechnology, Inc.) and 0.25 μM methotrexate as described previously (27). The cells were subcultured weekly, using standard tissue culture plastic ware. For most experiments, cells were grown in 100-mm culture dishes in complete medium until 85–100% confluent (2–3 days). For some experiments, subconfluent (50% confluent) cells were used (1–2 days). Cells were serum-starved for 18–24 h prior to experiments by replacing the medium with unsupplemented DME.

Protein Kinase Assays—The method used has been described previously (28). Briefly, serum-starved cells were incubated with insulin, phorbol 12-myristate 13-acetate (PMA), or lysophosphatidic acid by direct addition of the agents to the medium. Unless otherwise noted, 100 nM PMA (LC Services), 100 nM recombinant human insulin (generously gift of Lilly), or 10 μM 1-α-oleoyl-lysophosphatidic acid (LPA; Sigma) was used. Ethanol (0.1%) was included as a vehicle control for cells not receiving PMA. Cells were then incubated at 37 °C in a cell culture incubator. The incubation was terminated by removing the medium, washing twice with 1 ml of ice-cold Dulbecco’s phosphate-buffered saline, and then scraping the cells into 1 ml of ice-cold phosphatidylserine (Sigma) to be homogenized in the presence of 0.1% ethanol. Following the incubation, cellular lipids were extracted and resolved by thin-layer chromatography. [3H]Phosphoric acid (PA) and [3H]phosphatidylethanolamine (PET) were quantitated by liquid scintillation spectrometry.

MAPK Activity—The methods used to measure phospholipase D (PLD) activity in intact cells and membrane preparations have been described previously (28, 30). Briefly, for the intact cell assay, confluent Rat-1 cells grown in 35-mm dishes were cultured for 18 h in 2 ml of DME containing 5 μCi/ml [3H]palmitic acid (Dupont NEN). Quadruplicate dishes were used for each experimental condition. Washed cells were incubated with 1 μM insulin or 100 nM PMA for 15 min at 37 °C in the presence of 0.5% ethanol. Following the incubation, cellular lipids were extracted and resolved by thin-layer chromatography. [3H]Phosphatic acid (PA) and [3H]phosphatidylethanolamine (PET) were quantitated by liquid scintillation spectrometry.

Immunoprecipitation—Whole cell extracts were prepared as described above, except that the lysis buffer used contained 20 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Proteins were resolved on Laemmli SDS-PAGE gels (10, 12.5, or 15%) and then transferred to poly(vinylidene fluoride) membranes. Primary antibodies to MAPK (pan-ERK; monoclonal), Shc (polyclonal), Grb2 (monoclonal), FAK (monoclonal), and phosphotyrosine (PY20; monoclonal) were obtained from Transduction Laboratories. The pan-ERK antibody recognizes the ERK1 and ERK2 isoforms of MAPK. After incubation with primary antibodies, immunoblots were often used to measure the phosphorylation state of MAPK in intact cells and membrane preparations.

Immunoblotting—Whole cell extracts were prepared as described above for immunoblotting. Antibodies to Shc were incubated with the extracts for 2.5 h at 4 °C with mixing. Protein A-agarose was then added, and the incubation was continued for 1 h. The agarose beads were collected by centrifugation, washed with buffer, and then boiled in Laemmli sample buffer. Anti-ERK immunoprecipitations were carried out as described above, except that cells were extracted with boiling 1% SDS, 10 mM Tris-HCl (pH 7.4) and then sonicated (denaturing conditions). Dephosphorylation of immunoprecipitated Shc was carried out as described previously by Waters et al. (29); immunoprecipitated MAPK was phosphorylated in vitro in the presence of [32P]ATP and then immunoprecipitated with an antibody to MAPK. Immunoprecipitated MAPK was then assayed by immunoblotting with monoclonal antibodies to MAPK. Immunoblot analyses were used to measure the phosphorylation state of MAPK in intact cells and membrane preparations. Immunoblot analysis of MAPK in intact cells and membrane preparations was often used to measure the phosphorylation state of MAPK in intact cells and membrane preparations.
Values are expressed as a percent of the total radioactivity recovered from each dish.

RESULTS

Effects of Insulin, Phorbol Ester, and LPA on MAPK Activity—The effects of insulin and PMA on MAPK activity in Rat-1 HIR cells were examined. MAPK and RSK activities were initially measured in cytosolic extracts prepared from cells treated with insulin or PMA, using MBP as substrate. Either insulin or PMA alone induced modest increases in MBP phosphorylation in confluent cells (Fig. 1A). A combination of insulin and PMA induced a profound and synergistic activation of MBP phosphorylation. The order of agonist addition was not critical. Similar results were seen for phosphorylation of S6 peptide, a substrate for RSK (Fig. 1B). The extent of activation of MAPK by insulin and PMA alone was variable between experiments, with no significant activation seen in some cases. The results from 24 experiments, in which 100 nM PMA and insulin were incubated with cells for 5 min, were calculated as follows: \[\frac{(P + I) - C}{(P - C) + (I - C)}\]. In other words, MBP kinase activity from untreated cells (C), which can include the activity of kinases other than MAPK, was subtracted from the activities obtained with either PMA (P), insulin (I), or PMA plus insulin (P + I). The activity seen in the presence of both agonists, divided by the sum of the activities seen in response to either agonist alone, gives a value expressing the observed activity as a ratio of the activity expected if the effects of the two agents were additive. The value obtained was 3.43 ± 0.50 (mean ± S.E., n = 24, p < 0.001), indicating that the observed activity was more than 3-fold higher than that expected. These results show that, in confluent Rat-1 HIR cells, insulin and phorbol ester can act synergistically to activate MBP kinase activity.

Since insulin has previously been reported to markedly activate MAPK in Rat-1 HIR cells (27, 31), a number of experimental variables were examined in order to identify factors contributing to the relative insulin resistance observed in our studies. Insulin or phorbol ester alone activated cytosolic MBP kinase activity by less than 3-fold in most experiments (Fig. 2). MBP kinase activity measured in detergent-solubilized membrane fractions was activated in the same manner as that measured in cytosol (data not shown). Factors potentially contributing to variations in responsiveness were examined. These included composition of the cell lysis buffer (e.g., inclusion of additional inhibitors of serine/threonine and tyrosine phosphatases), passage number of the cells, presence of glucose in the incubation medium, presence of serum (with and without serum starvation), duration of serum starvation (1–5 days), starvation conditions (DME versus DME/F-12 medium), and state of confluence of the cells. None of these factors was found to consistently increase MBP kinase activation in response to insulin or PMA alone. Similar responses were seen in the absence or presence of serum for both subconfluent cells (Fig. 2) and confluent cells (Fig. 2 and data not shown).

In order to confirm that the activated MBP kinase activity represented MAPK (i.e., ERKs), cytosolic proteins were partially purified by Mono Q chromatography. Three peaks of MBP kinase activity were resolved (Fig. 3A). Using immunoblotting with a pan-ERK antiserum, immunoreactive proteins were observed in column fractions containing MBP kinase activity (Fig. 3B). The first peak, which was the major peak with respect to both kinase activity and immunoreactivity, contained an immunoreactive protein of approximately 42 kDa. This protein probably represents ERK2, which elutes at this salt concentration under our chromatography conditions. The second peak (~45 kDa) is an unidentified MAPK isoform. The third peak (~44 kDa) likely represents ERK1, which elutes here under the conditions used. These results indicate that the MBP kinase activity that is synergistically stimulated by PMA and insulin represents MAPK isoforms.

The ability of PMA and insulin to induce phosphorylation of MAPK was studied. MAPK was immunoprecipitated and then subjected to immunoblotting for MAPK and phosphotyrosine
ERK2 was detected as a 42-kDa band in the immunoprecipitates. An additional band of lower mobility, labeled ERK2\(^{9}\), was detected in cells treated with PMA, insulin, or PMA plus insulin. Shifts in the electrophoretic mobility of MAPK are typically seen following its phosphorylation and activation. The combination of insulin and PMA caused a more complete shift in MAPK mobility than was seen for either agent alone. Phosphotyrosine was detected in a band that co-migrated with the shifted band of ERK2 seen in cells treated with both PMA and insulin, but was not detected in ERK2 from cells treated with insulin or PMA alone. The results shown in Fig. 3C confirm that co-application of insulin and PMA results in enhanced activation of MAPK.

The time course of the response to insulin and PMA, alone and in combination, was examined. Combined data from two experiments in which partial activation of MAPK was seen in response to insulin and PMA are shown in Fig. 4A. Activation of MAPK was maximal within 10 min in response to either insulin or PMA alone. The response to the combination of insulin and PMA followed a similar time course. These results show that the synergistic effect increases the magnitude of the response, rather than its duration.

The duration and magnitude of agonist-induced MAPK activation can be regulated by induction of dual-function MAPK phosphatases, some of which are encoded by immediate-early genes (32, 33). Inhibition of phosphatase induction by one agent could potentially enhance responsiveness to another. The time course of the response was measured in the absence and presence of actinomycin D (Fig. 4B). The response to insulin plus PMA was similar, at 5–10-min incubation, in the presence or absence of actinomycin D. The responses to insulin or PMA alone, which were negligible in this experiment, were not enhanced by actinomycin D. These data suggest that rapid induction of a phosphatase (or other protein) is not involved in regulating the magnitude of the acute responses to insulin, PMA, or insulin plus PMA. Thus, the observed synergism, which was routinely detected at 5 min, was not mediated by effects of insulin or PMA on inducible phosphatases.

Concentrations of 100 nM insulin and 100 nM PMA were generally used in these studies. The dose-response relationships for insulin and PMA were examined (data not shown). The EC\(_{50}\) values for the effects of insulin and PMA on MAPK
The effects of LPA, a mitogenic agonist for fibroblasts, were also examined (Fig. 5). LPA alone did not significantly increase MAPK activity, but enhanced MAPK activation in response to insulin. The synergistic effect of LPA was usually less than that observed for PMA. In experiments in which the synergistic responses to LPA and PMA were similar, and in which PMA alone caused significant activation of MAPK, there was still no significant response to LPA alone (see Fig. 7). Similar effects were seen in subconfluent and confluent cells (data not shown). Thus, while LPA alone does not significantly activate MAPK in these cells, its ability to synergistically enhance MAPK activation mimics that of phorbol ester.

Effects of Insulin and Phorbol Ester on PLD Activity—To determine whether insulin modulated PKC activation, we examined activation of phospholipase D. PLD is generally activated by PMA, while effects of insulin on PLD have not been well established. Intact cells were incubated with insulin and PMA in the presence of ethanol. PMA stimulated PLD activity as measured by production of both PEt and PA (Fig. 6). Insulin caused a partial activation of PLD. The combination of insulin and PMA resulted in PEt levels similar to those seen with PMA alone. Similar results were seen with an in vitro assay for PLD activity (30), using membranes prepared from control and agonist-treated cells (data not shown). LPA did not activate PLD in intact cells and did not affect activation of PLD in response to insulin (Fig. 6) or PMA (data not shown). The failure of insulin to augment PMA-mediated PLD activation suggests that insulin does not enhance activation of PKC.

Effects of Insulin and Phorbol Ester on Tyrosine Phosphorylation—We next examined whether the synergism between insulin and PMA could be detected at a step upstream of MAPK. As shown in Fig. 7, insulin or PMA induced shifts in mobility of a portion of the total immunoreactive MAPK. In cells incubated with both insulin and PMA, both MAPK isoforms shifted completely to the lower mobility forms. These results, together with the results shown in Figs. 1–3, confirm that both ERK1 and ERK2 are more fully activated in response to a combination of insulin and PMA than in response to either agent alone. In the same experiment, the mobility of Shc was examined. Some forms of Shc show shifts to a lower mobility form when phosphorylated on tyrosine (34). We observed shifts in the mobility of the 52- and 66-kDa forms of Shc in response to PMA, insulin, and LPA (Fig. 7). The 46-kDa form of Shc did not exhibit mobility shifts. In the experiment shown in the upper blot, the effects of PMA on MAPK activity are typically ~3 nM. Since typical effective doses of both PMA and insulin were required for synergistic activation of MAPK, these results suggest that one agent does not alter the dose-response relationship for the other.

We examined whether PMA enhanced responsiveness to insulin, or vice versa, reasoning that a "priming" effect might be detected subsequent to treatment with insulin or PMA. When PMA was added 10 min prior to insulin, MAPK activity was similar to that seen when both agents were added at the same time (Fig. 5). When insulin was added 10 min prior to PMA, MAPK activation was reduced as compared to simultaneous addition. Little synergism was noted when PMA was added 30 or 60 min prior to insulin (data not shown). These data suggest that PMA enhances the ability of insulin to activate MAPK.

Thus, while LPA alone does not significantly activate MAPK in these cells, its ability to synergistically enhance MAPK activation mimics that of phorbol ester.

Panel A

In times with 100 nM insulin, 100 nM PMA, or a combination of insulin and PMA. MAPK activity was assessed in cytosolic extracts prepared from the cells. Each point represents the mean ± S.D. of values obtained from two separate experiments that were each performed with duplicate dishes of cells. In Panel B, an experiment was performed as described for Panel A, except that the cells were incubated with insulin, PMA, or insulin and PMA for 5 or 30 min in the absence or presence of 5 μg/ml actinomycin D. Actinomycin D was added to the cells 5 min prior to the addition of insulin and/or PMA. Each point represents the mean ± S.D. of values obtained from duplicate dishes of cells.

Fig. 4. Time course of the effects of agonists on MAPK activity. In Panel A, confluent Rat-1 HIR cells were incubated for the indicated times with 100 nM insulin, 100 nM PMA, or a combination of insulin and PMA. MAPK activity was assessed in cytosolic extracts prepared from the cells. Each point represents the mean ± S.D. of values obtained from two separate experiments that were each performed with duplicate dishes of cells. In Panel B, an experiment was performed as described for Panel A, except that the cells were incubated with insulin, PMA, or insulin and PMA for 5 or 30 min in the absence or presence of 5 μg/ml actinomycin D. Actinomycin D was added to the cells 5 min prior to the addition of insulin and/or PMA. Each point represents the mean ± S.D. of values obtained from duplicate dishes of cells.

activity could not be accurately determined, due to the relatively low responses. The synergistic effects of insulin and PMA (100 nM) were observed with 10 nM and 1 μM insulin, but not with 1 nM insulin. The EC50 for insulin in the presence of 100 nM PMA was approximately 3 nM. This value is consistent with the dose-response relationship observed for other insulin responses in these cells (3). MAPK activation was not observed with 1 nM PMA plus 10 nM insulin. Full activation was observed with 10 nM or 1 μM PMA plus 100 nM insulin. EC50 values for effects of PMA on MAPK activity are typically ~3 nM. Since typical effective doses of both PMA and insulin were required for synergistic activation of MAPK, these results suggest that one agent does not alter the dose-response relationship for the other.
forms of Shc. Similar results were seen for Shc from insulin-treated cells (data not shown). The data shown indicate that the lower mobility bands seen in our experiments represent phosphorylated Shc.

To further investigate this phenomenon, the effects of insulin and PMA on tyrosine phosphorylation were examined (Fig. 8A). As described previously for Rat-1 HIR cells, insulin induced phosphorylation of an ~90-kDa protein corresponding to the size of the insulin receptor β subunit. Phosphorylation of the insulin receptor was not induced by PMA. In some experiments, PMA caused a slight decrease in insulin-induced receptor phosphorylation, consistent with previous reports (24). Like PMA, LPA had no effect on the tyrosine phosphorylation state of the insulin receptor (data not shown). In some experiments, phosphorylation of a protein migrating at ~185 kDa, which probably represents IRS-1, was observed in response to insulin or PMA plus insulin (data not shown). These results suggest that PMA does not significantly alter the ability of insulin to phosphorylate IRS-1. Insulin caused a decrease in the phosphorylation state of a protein migrating at approximately 120 kDa (Fig. 8A), corresponding to the molecular size of FAK. Migration of FAK at this position was confirmed by immunoblotting with anti-FAK antibodies (data not shown). This decrease in FAK phosphorylation was not seen when cells were incubated with a combination of PMA and insulin. Similar results were seen for a combination of LPA and insulin (data not shown). These data establish that enhancement of MAPK activation by PMA is not due to enhancement of tyrosine phosphorylation of the insulin receptor, but suggest that co-incubation with PMA may block the ability of insulin to induce dephosphorylation of FAK.

The effects of insulin and PMA on the tyrosine phosphorylation of Shc were next examined. As shown in Fig. 8B (left), insulin alone induced tyrosine phosphorylation of the major 52-kDa species of Shc. Phosphotyrosine was also present, at barely detectable levels, in the 46-kDa form of Shc. Tyrosine phosphorylation of the 66-kDa form of Shc was not detected, but was likely below the level of detection of our assay. PMA (Fig. 8B, right) and insulin (data not shown) caused the usual shifts in mobility of the 52- and 66-kDa forms of Shc in this experiment, as detected in whole-cell extracts. The combination of insulin and PMA did not enhance tyrosine phosphorylation of 52-kDa Shc over that seen with insulin alone. Similar results were seen for LPA plus insulin (data not shown). When PMA was added 10 min prior to insulin, tyrosine phosphorylation of Shc was similar to that seen when insulin was added first. These results indicate that only insulin induces phosphorylation of Shc on tyrosine. Therefore, PMA-induced Shc phosphorylation (Fig. 7) likely occurs on serine/threonine residues.

The association of Shc with Grb2 was also examined (Fig. 8C). The amount of Grb2 detected in anti-Shc immunoprecipitates was increased by insulin, but not by PMA (Fig. 8C) or LPA (data not shown). The amount of Grb2 present in immunoprecipitates from cells incubated with PMA plus insulin, or LPA plus insulin, was similar to that seen from cells incubated with insulin alone. Taken together, these results indicate that insulin promotes the association of Shc with Grb2, but that PMA does not further enhance this association.

**DISCUSSION**

In these studies, we have shown that insulin and phorbol ester can cause a synergistic activation of MAPK in Rat-1 HIR cells. The current study used confluent, serum-starved cells, as have other studies of MAPK activation in Rat-1 cells (27, 31). Synergistic activation of MAPK by PMA and insulin was observed in both quiescent and proliferating cells (Fig. 2). It is not
clear why greater insulin-induced MAPK activation was detected in these cells in previous studies (27, 31). Differences may include variations between Rat-1 HIR cell lines, differences in assay conditions, or other factors. The results are consistent with a previous report that PMA enhances the ability of insulin to induce MAPK activation and mitogenesis in Balb/c 3T3 fibroblasts expressing the human insulin receptor (5), but are in contrast with PMA-induced inhibition of insulin-stimulated Shc tyrosine phosphorylation and mitogenesis observed in Chinese hamster ovary cells transfected with the human insulin receptor and PKCa (35). Thus, responses may vary with cell type, levels of PKC isoform expression, and levels of insulin receptor expression.

One interpretation of the synergistic effects of insulin and PMA is that two separate signaling pathways must be engaged for maximal MAPK activation. Insulin activates one of these pathways, while PMA activates the other. It is clear that insulin and PMA elicit different responses in confluent Rat-1 cells. For example, PMA causes changes in cell shape (data not shown) and activates PLD. These effects are either absent or reduced, respectively, in response to insulin.

A second interpretation of our results is that PMA can enhance insulin responsiveness via direct effects on the insulin receptor. Although phosphorylation of the receptor by PKC reduces its tyrosine kinase activity (24), this effect is not necessarily inhibitory to MAPK activation. Since removal of some of the tyrosine phosphorylation sites on the receptor enhances its ability to activate MAPK (3), it is possible that phosphorylation of the receptor by PKC alters effector binding to the receptor to favor MAPK activation. However, this possibility seems unlikely in view of our observation that LPA, which unlike PMA does not activate PLD and MAPK when added alone, can also act synergistically with insulin.

A third possibility is that PMA alters the abilities of effector proteins to interact with the insulin receptor and/or to activate the MAPK pathway. Our study provides at least two observations suggesting that this is the case. First, PMA enhances phosphorylation of Shc on serine/threonine residues. Since either insulin alone or PMA plus insulin induces similar binding of Grb2 to Shc, it appears that the enhanced phosphorylation of Shc observed in response to PMA plus insulin may play additional regulatory roles. In a previous study, an insulin receptor mutant with severely impaired ability to phosphorylate Shc on tyrosine was nonetheless able to partially activate Ras (36). These investigators suggested that further enhancement of Shc tyrosine phosphorylation could result in synergistic activation of Ras. Our data suggest that serine/threonine phosphorylation of Shc may also play a role in regulating Shc activity. While an increase in tyrosine phosphorylation of Shc, or of binding of Grb2 to Shc, was not detected in cells treated with both PMA and insulin, it is likely that serine/threonine phosphorylation enhances the interaction of Shc with receptors or effectors (25). The role of Shc in insulin signaling is underscored by a recent report showing that epidermal growth factor and insulin preferentially phosphorylate different forms of Shc (37). Second, our results indicate that PMA can counteract the dephosphorylation of FAK observed in response to insulin. Since the latter response appears to be deleterious for MAPK activation (16), this effect of PMA may represent one aspect of its synergistic activity. In fact, since phosphorylated FAK has been shown to bind Grb2 (38), maintenance or enhancement of FAK phosphorylation may represent a second "pathway" leading to MAPK activation in these cells.

Activation of PLD has been proposed to play a role in mitogenesis by providing a sustained source of diglyceride for PKC activation, via conversion of PA to diglyceride by PA phospholipase. LPA, which can be produced from PA via the action of a PA-utilizing phospholipase A2, is another potential mes-

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**Fig. 6.** Effects of insulin and PMA on PLD activity in confluent cells. Confluent Rat-1 HIR cells were incubated for 18 h with [3H]palmitic acid to label cellular lipid. The cells were then incubated for 15 min in the presence of 0.5% ethanol with 100 nm insulin, 100 nm PMA, 10 μM LPA, insulin and PMA, or insulin and LPA. Production of [3H]phosphatidylethanol was assessed following separation of extracted phospholipids by thin layer chromatography. Each data point represents the mean ± S.D. of values obtained from quadruplicate dishes of cells.
senger produced in response to activation of PLD. LPA induces stress fiber formation and activates FAK in fibroblasts (39). PA can promote actin polymerization in fibroblasts (40); this response could be mediated by LPA. Although LPA did not activate PLD in Rat-1 HIR cells, in contrast to results obtained by other investigators using Rat-1 fibroblasts (41), it acted synergistically with insulin to activate MAPK. The role of LPA as an autocrine factor produced via PLD activation deserves further consideration.

We have shown that insulin can cause a partial activation of PLD in intact Rat-1 HIR cells. The mechanism responsible for this effect has not yet been identified. Activation of PLD by insulin may either reflect direct coupling of the insulin receptor to an effector involved in PLD activation (e.g. rho), or may be subsequent to activation of PKC. For the platelet-derived growth factor receptor, activation of phosphatidylinositol-phospholipase C is necessary for PLD activation, while interaction of the receptor with Ras GTPase-activating protein, phosphati-

**FIG. 7. Effects of agonists on the electrophoretic mobilities of MAPK and Shc.** In Panel A, whole-cell extracts were prepared from confluent Rat-1 HIR cells that had been incubated for 10 min with no addition (C), 100 nM insulin (I), 100 nM PMA (P), 10 μM LPA (L), PMA plus insulin (P + I), or LPA plus insulin (L + I). Duplicate dishes of cells were used for each experimental condition. Proteins were resolved by SDS-PAGE on 12.5% Laemmli gels. For the experiment shown in the upper blot, the lower molecular size markers were run off the bottom of the gel to enhance separation of MAPK and Shc species. In the upper blot, immunoblotting was carried out for the upper portion of the gel using an anti-Shc antibody, and for the lower portion using a polyclonal pan-ERK antibody. The positions of the molecular size markers (lanes) are indicated on the right. In the lower blot, a replicate experiment is shown for immunoblotting with anti-Shc. Within each experiment, equivalent amounts of cellular protein were loaded on each lane. In Panel B, Shc was immunoprecipitated from cells treated for 10 min with no addition (C), 100 nM PMA (P), or PMA plus 100 nM insulin (P + I). The immunoprecipitates, shown on the left-hand side of the blot, were incubated with (+) and without (−) calf intestinal phosphatase (p’tase) as described in the text. The right-hand side of the blot contains whole-cell extracts prepared from cells incubated with no addition, PMA, 100 nM insulin (I), or PMA plus insulin (P + I). Proteins were resolved on 12.5% gels. The transferred gels were immunoblotted with anti-Shc.

**FIG. 8. Effects of agonists on tyrosine phosphorylation and Shc-Grb2 association in Rat-1 HIR cells.** Confluent Rat-1 HIR cells were incubated with no additions (C), 100 nM insulin (I), 100 nM PMA (P), 10 μM LPA (L), PMA plus insulin (P + I) or LPA plus insulin (L + I) for 10 min. The positions of the molecular size markers are indicated on the right. In all cases, each lane contains extracts or immunoprecipitates prepared from equivalent numbers of cells. In Panel A, whole cell extracts were prepared, and proteins were resolved on 12.5% SDS-PAGE gels. Lanes containing molecular size markers are indicated by s. Immunoblotting was performed using anti-phosphotyrosine. In Panel B, Shc immunoprecipitates were prepared from cells treated as described in Panel A. In addition, some cells were incubated for 10 min with one agent, followed by addition of the second agent for an additional 10 min (P, I = PMA, then insulin; I, P = insulin, then PMA). The immunoprecipitates were subjected to immunoblotting for phosphotyrosine. In Panel B, Shc immunoprecipitates were prepared from cells treated as described in Panel A. In addition, some cells were incubated for 10 min with one agent, followed by addition of the second agent for an additional 10 min (P, I = PMA, then insulin; I, P = insulin, then PMA). The immunoprecipitates were subjected to immunoblotting for phosphotyrosine. In the far right lane of the blot, a whole-cell extract from PMA-treated cells was immunoblotted for Shc. In Panel C, anti-Shc immunoprecipitates were prepared as described for Panel B; proteins were resolved on 12.5% SDS-PAGE gels. The gels were immunoblotted using anti-Grb2. The lane on the far right contains whole-cell extract prepared from cells treated with LPA plus insulin. Equivalent immunoprecipitation of Shc was confirmed by immunoblotting the upper portion of the gel for Shc (data not shown).
dylinositol 3-kinase, or Syp is not required (42). Likewise, epidermal growth factor-induced activation of PLD is dependent on activation of phosphatidylinositol-phospholipase C and PKC in fibroblasts (43).

It is likely that PMA and LPA enhance responsiveness to insulin by activating a separate signaling pathway(s). The lack of enhancement of insulin’s effects on Shc-Grb2 association by PMA (Fig. 8) suggest that this is the case. Cooperative activation of MAPK by α2-adrenergic agonists and PMA was recently reported in Chinese hamster ovary cells; the α2α-adrenergic response was Ras-independent (44). Two independent signals, one of which is PKC-dependent, are needed for maximal activation of MAPK by the G-protein-coupled anaphylatoxin C5a receptor (45). Recently, activation of MAPK by LPA in Rat-I cells was shown to be mediated by both G proteins and ras, but to be independent of PKC (46). These data support the recent demonstration that more than one signaling pathway can contribute to MAPK activation (47), and are consistent with the suggestion that the effects of PKC activation on MAPK can likely be mediated at several upstream sites in the MAPK cascade (48).

The results described herein suggest that phorbol esters can sensitize cells to the mitogenic effects of growth factors. This capability potentially represents a significant feature of the tumor-promoting activity of phorbol esters.

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