Differential Regulation of the Phosphatidylinositol 3-Kinase/Akt and p70 S6 Kinase Pathways by the α1A-Adrenergic Receptor in Rat-1 Fibroblasts*

(Received for publication, September 8, 1999, and in revised form, November 24, 1999)

Lisa M. Ballou‡, Michael E. Cross‡, Siqi Huang‡, E. Michael McReynolds‡, Bin-Xian Zhang§, and Richard Z. Lin‡||

From the Departments of ¶Pharmacology and ¶Medicine, University of Texas Health Science Center at San Antonio and the §§Research Service, Audie L. Murphy Memorial Veterans Hospital, San Antonio, Texas 78284

Phosphatidylinositol (PI) 3-kinase and its downstream effector Akt are thought to be signaling intermediates that link cell surface receptors to p70 S6 kinase. We examined the effect of a Gq-coupled receptor on PI 3-kinase/Akt signaling and p70 S6 kinase activation using Rat-1 fibroblasts stably expressing the human α1A-adrenergic receptor. Treatment of the cells with phenylephrine, a specific α1-adrenergic receptor agonist, activated p70 S6 kinase but did not activate PI 3-kinase or any of the three known isoforms of Akt. Furthermore, phenylephrine blocked the insulin-like growth factor-I (IGF-I)-induced activation of PI 3-kinase and the phosphorylation and activation of Akt-1. The effect of phenylephrine was not confined to signaling pathways that include insulin receptor substrate-1, as the α1-adrenergic receptor agonist also inhibited the platelet-derived growth factor-induced activation of PI 3-kinase and Akt-1. Although increasing the intracellular Ca2+ concentration with the ionophore A23187 inhibited the activation of Akt-1 by IGF-I, Ca2+ does not appear to play a role in the phenylephrine-mediated inhibition of the PI 3-kinase/Akt pathway. The differential ability of phenylephrine and IGF-I to activate Akt-1 resulted in a differential ability to protect cells from UV-induced apoptosis. These results demonstrate that activation of p70 S6 kinase by the α1A-adrenergic receptor in Rat-1 fibroblasts occurs in the absence of PI 3-kinase/Akt signaling. Furthermore, this receptor negatively regulates the PI 3-kinase/Akt pathway, resulting in enhanced cell death following apoptotic insult.

Cellular growth requires the generation of new translational machinery to accommodate the increased demand for additional proteins. It has been shown that treatment of cells with growth-promoting agents induces the translational up-regulation of ribosomal proteins and protein synthesis elongation factors (1, 2). This process is controlled in part by phosphorylation of the S6 protein of 40 S ribosomal subunits by the Mr = 70,000 S6 kinase (p70 S6 kinase; Refs. 3 and 4). p70 S6 kinase is activated upon treatment of cells with a variety of growth factors, hormones, mitogens, and phosphatase inhibitors, etc. This increase in activity is due to phosphorylation of p70 S6 kinase at multiple sites presumably by multiple kinases (5, 6). Due to its importance in the growth response, the signal transduction pathways leading to activation of p70 S6 kinase have received considerable attention. A variety of experimental approaches have led to the identification of phosphatidylinositol (PI) 3-kinase and its downstream effector, the protein kinase Akt, as signaling intermediates that link cell surface receptors to p70 S6 kinase. First, treatment of cells with wortmannin or LY294002, two inhibitors of PI 3-kinase, prevents the activation of Akt (7–9) and p70 S6 kinase (10, 11) in response to growth factors or hormones. Second, platelet-derived growth factor (PDGF) receptor mutants that cannot bind PI 3-kinase were unable to induce efficiently the activation of Akt (7, 8) or p70 S6 kinase (12, 13). Third, overexpression of constitutively active forms of PI 3-kinase induces the activation of Akt (14–16) and p70 S6 kinase (15–17) in the absence of added extra-cellular ligands. Conversely, expression of dominant-negative mutants of the p85 subunit of PI 3-kinase inhibits the PDGF-induced activation of Akt (7) and p70 S6 kinase (12). Finally, overexpression of a dominant-negative mutant of Akt causes a reduction in insulin-induced activation of p70 S6 kinase (18), whereas constitutively or conditionally active forms of Akt stimulate p70 S6 kinase (7, 8, 16, 19, 20). Together, these results suggest the existence of a linear signaling pathway leading from receptors to PI 3-kinase, Akt and p70 S6 kinase. This pathway is also thought to involve additional components, as Akt does not phosphorylate p70 S6 kinase directly in vitro (21).

Like p70 S6 kinase, Akt is activated by a wide variety of hormones, growth factors, and other stimuli (7, 9, 22). Akt is thought to mediate many of the cellular effects of insulin and insulin-like growth factor-I (IGF-I) on glucose metabolism and cell survival (23, 24). For example, Akt phosphorylates and inactivates glycogen synthase kinase-3 in cells treated with insulin or IGF-I, thus promoting glycogen synthesis (25). Similarly, the anti-apoptotic effect of insulin and IGF-I is partly mediated by Akt phosphorylation of the pro-apoptotic protein BAD (26, 27). Akt is activated by phosphorylation of Thr-308 in the activation loop of the catalytic domain and Ser-473 in the carboxyl-terminal tail (22). It is believed that phosphorylation of both sites requires an interaction between the amino-termi

* This work was supported by a grant-in-aid from the American Heart Association, Texas Affiliate, Inc. (to L. M. B.), and by the Pharmaceutical Research and Manufacturers of America Foundation (to R. Z. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, Mail Code 7764, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., San Antonio, TX 78284-3900. Tel.: 210-567-2978; Fax: 210-567-4303; E-mail: lmir@uthscsa.edu.

§ The abbreviations used are: p70 S6 kinase, Mr = 70,000 ribosomal protein S6 kinase; IGF-I, insulin-like growth factor-I; PDGF, platelet-derived growth factor; PKC, protein kinase C; PI, phosphatidylinositol; PE, phosphylinositol [Ca2+]i, intracellular Ca2+ concentration; AR, adrenergic receptor; PMA, phorbol 12-myristate 13-acetate; IRS-1, insulin receptor substrate-1; HPLC, high pressure liquid chromatography.
nal pleckstrin homology domain of Akt with membrane inositol phospholipids generated by PI 3-kinase (23, 24, 28). Translocation of Akt to the membrane is thought to induce a conformational change that permits phosphorylation of Thr-308 and Ser-473 by membrane-associated Akt kinases. The kinase that phosphorylates Thr-308 in Akt has been identified as 3-phosphoinositide-dependent protein kinase 1 (29, 30). 3-Phosphoinositol-dependent protein kinase 1 also phosphorylates the equivalent site in some other protein kinases, including p70 S6 kinase (21, 31).

In contrast to insulin and IGF-I, treatment of cells with catecholamines to activate α1-adrenergic receptors (ARs) induces glycosylation. Three α1-AR subtypes have been cloned (α1A, α1B, and α1D), and all three receptors activate phospholipase C β, which promotes increased production of inositol 1,4,5-trisphosphate and diacylglycerol, leading to elevation of the intracellular Ca2⁺ concentration ([Ca2⁺]i) and activation of protein kinase C (PKC), respectively (32). Recent evidence indicates that α1-ARs also stimulate additional physiologically relevant signaling pathways. For example, treatment of cultured rat neonatal cardiac myocytes with the α1-AR agonist phenylephrine (PE) leads to activation of p70 S6 kinase and an increase in the rate of protein synthesis and cell growth (33).

Since activation of Akt promotes glycogen synthesis, it seemed inconsistent that α1-ARs, which stimulate glycogen breakdown, would signal to p70 S6 kinase via an Akt-dependent pathway. In this study, we examined this question using Rat-1 fibroblasts expressing the human α1A-AR. We show that treatment of these cells with PE activates p70 S6 kinase but does not activate PI 3-kinase or Akt. Moreover, we show that the α1A-AR inhibits the activation of PI 3-kinase and Akt induced by other growth factors. Finally, we tested whether the differential ability of PE and IGF-I to activate Akt correlates with their ability to protect cells from UV-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—PE and IGF-I were from Sigma. Human recombinant PDGF A/B was from Roche Molecular Biochemicals. [γ-32P]ATP (3000 Ci/mmol) was from NEN Life Science Products. PI was purchased from Avanti Polar Lipids (Alabaster, AL). Phospho-specific Akt-1 antibodies were from New England Biolabs (Beverly, MA). A23187, rapamycin, LY294002, and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem. All other reagents of molecular biology grade were obtained from standard commercial suppliers.

Cell Culture—Rat-1 fibroblasts stably transfected with the human α1A-AR were a gift from Dr. G. Johnson of Pfizer (34). Cells were maintained in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) with 10% fetal bovine serum (Sigma) in 5% CO2 at 37 °C. Rat-1 fibroblasts stably transfected with the human α1A-AR were seeded at 3 × 10⁴ cells/cm², and all three receptors activate phospholipase C β, which promotes increased production of inositol 1,4,5-trisphosphate and diacylglycerol, leading to elevation of the intracellular Ca2⁺ concentration ([Ca2⁺]i) and activation of protein kinase C (PKC), respectively (32). Recent evidence indicates that α1-ARs also stimulate additional physiologically relevant signaling pathways. For example, treatment of cultured rat neonatal cardiac myocytes with the α1-AR agonist phenylephrine (PE) leads to activation of p70 S6 kinase and an increase in the rate of protein synthesis and cell growth (33).

Since activation of Akt promotes glycogen synthesis, it seemed inconsistent that α1-ARs, which stimulate glycogen breakdown, would signal to p70 S6 kinase via an Akt-dependent pathway. In this study, we examined this question using Rat-1 fibroblasts expressing the human α1A-AR. We show that treatment of these cells with PE activates p70 S6 kinase but does not activate PI 3-kinase or Akt. Moreover, we show that the α1A-AR inhibits the activation of PI 3-kinase and Akt induced by other growth factors. Finally, we tested whether the differential ability of PE and IGF-I to activate Akt correlates with their ability to protect cells from UV-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—PE and IGF-I were from Sigma. Human recombinant PDGF A/B was from Roche Molecular Biochemicals. [γ-32P]ATP (3000 Ci/mmol) was from NEN Life Science Products. PI was purchased from Avanti Polar Lipids (Alabaster, AL). Phospho-specific Akt-1 antibodies were from New England Biolabs (Beverly, MA). A23187, rapamycin, LY294002, and phorbol 12-myristate 13-acetate (PMA) were from Calbiochem. All other reagents of molecular biology grade were obtained from standard commercial suppliers.

Cell Culture—Rat-1 fibroblasts stably transfected with the human α1A-AR were a gift from Dr. G. Johnson of Pfizer (34). Cells were maintained in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) with 10% fetal bovine serum (Sigma) in 5% CO2 at 37 °C. Before treatment, cells were incubated in serum-free medium for 16–18 h. For experiments involving Ca2⁺, the cells were preincubated for 1 h at 37 °C for 30–45 min (38). Loaded cells were washed with fresh buffer to remove the extracellular dye and resuspended in high salt glucose buffer containing either 1 mM Ca2⁺ or 100 μM EGTA. The 340/380 excitation ratio was measured with a PTI Delta Scan spectrophotometer (Photon Technology International, Inc., South Brunswick, NJ) using 340 and 380 nm excitation and 510 nm emission. [Ca2⁺], values were calculated according to the equation: [Ca2⁺]i (nm) = Kd[R(max)−[R(min)[R(max)−R(min)]−R], where R is the 340/380 fluorescent ratio; R(min) and R(max) are the minimal and maximal fluorescent ratios, respectively; and the Kd (dissociation constant) of fura-2 for Ca2⁺ is taken to be 224 nm (39). After a stable basal fluorescent ratio was established, the cells were subjected to various treatments.

Apoptosis—Rat-1 cells expressing the α1A-AR were seeded at 3 x 10⁵ cells per 10-cm dish. The next day the medium was removed, and the cells were serum-starved for 12 h and then pretreated with or without agonists for 5 min. The medium was then removed, and the cells were exposed to 10 μg/ml of 254 nm UV light, and the medium with or without agonists was replaced. Apoptotic cells were labeled 6 h after irradiation using the fluorescein-based In Situ Cell Death Detection kit (Roche Molecular Biochemicals) and analyzed by flow cytometry according to the manufacturer’s instructions.

RESULTS

Differential Activation of p70 S6 Kinase, PI 3-Kinase, and Akt by the α1A-AR—The Rat-1 fibroblasts used in this study stably express the human α1A-AR; they do not express endogenous α1-ARs (40). Treatment of the cells with PE, a selective α1-AR agonist, promoted an increase in S6 kinase activity as...
Rat-1 fibroblasts expressing the extracellular ligands. If this is the case, then PE treatment of upstream of p70 S6 kinase in signaling pathways activated by PI 3-kinase and its downstream effector Akt act requires PI 3-kinase but not PKC.

Akt immunocomplex kinase assays on lysates prepared for 30 min with 100 nM PE as described for A. The PE-induced increase in Akt activity. This hypothesis was tested by performing Akt immunocomplex kinase assays on lysates prepared from cells treated for 5 min with or without PE. Surprisingly, no increase in Akt-1 activity was detected in cells treated with PE (Fig. 2A). Exposure of cells to PE for a longer time (up to 30 min; see Fig. 5B) or at a higher concentration (100 μM; data not shown) did not lead to a measurable activation of Akt-1. By contrast, treatment with IGF-I caused a large increase (approximately 11-fold) in Akt-1 activity (Fig. 2A). To assess the possibility that the α1A-AR might activate one of the other two known isoforms of Akt, we also performed immunocomplex kinase assays of Akt-2 and Akt-3. The basal Akt-2 activity was about 4 times higher than that of Akt-1 or Akt-3 in untreated cells, and treatment with PE or IGF-I induced no further increase in Akt-2 activity (Fig. 2A). Little or no activation of Akt-2 by insulin has been observed in other cell types (43). Western blot analysis confirmed the presence of Akt-2 in these cells pretreated with PE (Fig. 2A).

As outlined in the Introduction, a variety of evidence suggests that PI 3-kinase/Akt and p70 S6 kinase signaling participates in this response. To resolve this apparent contradiction, PI 3-kinase activity was directly measured by analyzing the production of 3-phosphorylated phosphoinositides in cells treated with PE. Rat-1 fibroblasts grown in the presence of [3H]myo-inositol were treated with or without agonists and [3H]myo-inositol-labeled material recovered from the column. The amount of PI 3,4-bisphosphate and PI 3,4,5-trisphosphate recovered from cells treated for 5 min with or without PE. Surprisingly, no increase in Akt-1 activity was detected in cells treated with PE (Fig. 2A). Exposure of cells to PE for a longer time (up to 30 min; see Fig. 5B) or at a higher concentration (100 μM; data not shown) did not lead to a measurable activation of Akt-1. By contrast, treatment with IGF-I caused a large increase (approximately 11-fold) in Akt-1 activity (Fig. 2A). To assess the possibility that the α1A-AR might activate one of the other two known isoforms of Akt, we also performed immunocomplex kinase assays of Akt-2 and Akt-3. The basal Akt-2 activity was about 4 times higher than that of Akt-1 or Akt-3 in untreated cells, and treatment with PE or IGF-I induced no further increase in Akt-2 activity (Fig. 2A). Little or no activation of Akt-2 by insulin has been observed in other cell types (43). Western blot analysis confirmed the presence of Akt-2 in these cells pretreated with PE (Fig. 2A).
Effect of α₁A-AR on IGF-I-induced Akt Activation—Akt has been proposed to play an important role in insulin-induced glycogen synthesis (25). Since physiological stress induces a relatively insulin-resistant state, we wondered whether catecholamines might negatively regulate insulin signaling to Akt. We found that IGF-I-induced Akt-1 kinase activation was strongly suppressed in cells co-treated with PE (Fig. 3A). We found that IGF-I-induced Akt-1 kinase activation was strongly suppressed in cells co-treated with PE (Fig. 3A). Acti-

vation of Akt-1 is associated with phosphorylation of Thr-308 in the catalytic domain and Ser-473 in the carboxyl-terminal tail (22). We used Western blots probing with antibodies that specifically recognize these phosphorylated sites to examine further the activation state of Akt-1 in cells treated with IGF-I and PE. As shown in Fig. 3B (upper two panels), Akt-1 in untreated cells was not phosphorylated at either Thr-308 or Ser-473, and IGF-I strongly stimulated the phosphorylation of both residues. In contrast, cells treated with PE contained no detectable phospho-Thr-308 or phospho-Ser-473. In cells treated with IGF-I plus PE, the phosphate content of both residues was sharply reduced as compared with cells treated with IGF-I alone (Fig. 3B). The blot was stripped and reprobed with a general Akt-1 antibody to show that an equal amount of Akt-1 was present in each lane (Fig. 3B, lower panel). These results indicate that the α₁A-AR initiates a pathway that negatively regulates IGF-I-induced phosphorylation and activation of Akt-1.

Effect of α₁A-AR on PI 3-Kinase Activation—We next searched for an upstream component in the Akt-1 signaling pathway that might be a target for PE-induced inhibition. PI 3-kinase is thought to be a component in the pathway, so we measured the activity of this enzyme in phosphotyrosine immunoprecipitates. Consistent with the phospholipid analysis (Fig. 2B), treatment of cells with PE alone for 5 min caused a reduction in the basal level of PI 3-kinase activity (Fig. 4). By contrast, PI 3-kinase activity in cells treated with IGF-I alone was increased about 2-fold above the basal level. Finally, PE strongly antagonized the IGF-I-induced activation of PI 3-kinase, reducing PI 3-kinase activity almost to the level seen in control cells (Fig. 4). The behavior of PI 3-kinase in response to these cell treatments closely parallels that of Akt-1 kinase activity (Fig. 3A). Thus, the α₁A-AR appears to induce a negative regulatory mechanism that opposes Akt activation by inhibiting its upstream regulator, PI 3-kinase.

Increased serine phosphorylation of insulin receptor substrate-1 (IRS-1) has been shown to regulate negatively the activation of PI 3-kinase by insulin/IGF-I (44–46). We wondered whether the inhibitory effect of the α₁A-AR on PI 3-kinase is restricted to signaling pathways that include IRS-1 or whether it might be a more general phenomenon. Therefore, we tested the effect of PE on PI 3-kinase activation induced by PDGF, which does not signal through IRS-1. Cells were treated for increasing times with PDGF in the presence or absence of PE, and PI 3-kinase activity was measured in phosphotyrosine immunoprecipitates. PDGF was much more effective than IGF-I at activating PI 3-kinase in these cells; after 5 min in the presence of PDGF the PI 3-kinase activity increased approximately 55-fold over the basal level in untreated control cells (Fig. 5A). We were surprised that the PDGF-induced increase in 3-phosphorylated phosphoinositides in vivo (Fig. 2B) was relatively small in comparison to the large increase in PI 3-kinase activity measured in vitro (Fig. 5A). It could be that PI 3-kinase substrates are limiting in vivo or that 3-phosphorylated phosphoinositides produced in vivo are rapidly dephosphorylated. Activation of PI 3-kinase by PDGF was reduced approximately 50% in the presence of PE at the 5-min time point (Fig. 5A). At all times examined, PI 3-kinase activity was lower in cells treated with PDGF plus PE than in cells treated with PDGF alone (Fig. 5A). As a consequence of the inhibitory effect of PE on PI 3-kinase, PDGF-induced Akt-1 activity was also reduced in cells co-treated for 30 min with PE and PDGF (Fig. 5B). Thus, the inhibitory mechanism initiated by the α₁A-AR targets PI 3-kinase even in signaling pathways that do not include IRS-1.

Effect of [Ca²⁺]i on Akt-1 Activation—We have observed that PE treatment of Rat-1 fibroblasts expressing the α₁A-AR induces a sustained increase in [Ca²⁺], that is much larger than that produced by IGF-I (40). We therefore investigated whether Ca²⁺ might be involved in the PE-induced mechanism that inhibits activation of the PI 3-kinase/Akt-1 pathway. In the first experiment, cells were pretreated with or without the Ca²⁺ ionophore A23187 and then exposed to IGF-I to stimulate

References

1. Faingold, C. L., Lin, R. Z., and Saltiel, A. R. (1991) J. Biol. Chem. 266, 3147–3151
2. R. Z. Lin, unpublished data.
Akt-1. Consistent with an inhibitory role for high [Ca\(^{2+}\)], immunoprecipitation kinase assays showed that the IGF-I-induced activation of Akt-1 was strongly inhibited in cells pretreated with A23187 (Fig. 6A).

By having established that high [Ca\(^{2+}\)], seems to regulate Akt-1 activity negatively, we directly assessed whether the inhibitory effect of PE on IGF-I-induced activation of the kinase is mediated by Ca\(^{2+}\). Rat-1 fibroblasts were incubated in medium containing 1 mM Ca\(^{2+}\) or in Ca\(^{2+}\)-free medium containing 2 mM EGTA to deplete intracellular Ca\(^{2+}\) stores. The cells were then treated with or without agonists, and Akt-1 activity was measured in immunoprecipitates. Akt-1 activity in control and PE-treated cells was slightly reduced in cells incubated in Ca\(^{2+}\)-free versus Ca\(^{2+}\)-containing medium (Fig. 6B). IGF-I stimulation of Akt-1 was unaffected by the absence of intracellular Ca\(^{2+}\). Finally, depletion of intracellular Ca\(^{2+}\) only slightly alleviated the PE-induced inhibition of the IGF-I response (Fig. 6B). In a parallel control experiment, cells incubated in the presence or absence of Ca\(^{2+}\)-containing medium were stimulated with PE, and the intracellular Ca\(^{2+}\) response was measured by spectrofluorimetric analysis. As expected, cells incubated in the presence of Ca\(^{2+}\) had a higher basal [Ca\(^{2+}\)], than those incubated in Ca\(^{2+}\)-free medium (Fig. 6C). In addition, cells incubated in the presence of Ca\(^{2+}\) generated a robust release of intracellular Ca\(^{2+}\) in response to PE treatment, whereas those incubated in Ca\(^{2+}\)-free medium showed no Ca\(^{2+}\) response (Fig. 6C). Thus, although high [Ca\(^{2+}\)], appears to antagonize Akt-1 activation, the PE-induced inhibition of Akt-1 activation by IGF-I can occur even in the absence of intracellular Ca\(^{2+}\) release.

Effect of PE and IGF-I on UV-induced Apoptosis—Recent evidence indicates that the PI 3-kinase/Akt pathway delivers an anti-apoptotic signal (26, 27). We reasoned that PE and IGF-I, because of their differential ability to activate PI 3-kinase and Akt-1, would also show differential ability to protect cells from UV-induced apoptosis. To test this hypothesis, Rat-1 cells were preincubated with or without PE or IGF-I and then exposed to UV irradiation. TUNEL-positive cells were then quantitated 6 h later by flow cytometry. Approximately 3.5% of the cells in the control culture were apoptotic (Fig. 7). After exposure to UV light this value increased to 9%. PE potentiated the lethal effect of UV exposure, whereas cell cultures kept in the presence of IGF-I showed a reduced number of apoptotic cells both with and without PE treatment (Fig. 7). This biological response correlates well with the stimulatory and inhibitory effects of IGF-I and PE, respectively, on the PI 3-kinase/Akt pathway.

**DISCUSSION**

The results presented here demonstrate that activation of Akt is not required for activation of p70 S6 kinase. Treatment of Rat-1 cells expressing the \(\alpha_{1A}\)-AR with PE did not stimulate any of the three Akt isoforms even though there was a significant increase in p70 S6 kinase activity (Figs. 1 and 2). We further show that p70 S6 kinase can be activated even in cells in which PI 3-kinase is inhibited (Figs. 1A and 4). This result was unexpected because pharmacological approaches and co-expression studies using highly active or dominant-negative versions of these signaling molecules have indicated that PI 3-kinase and Akt are major upstream regulators of p70 S6 kinase (7, 8, 10–13, 15–17, 19). However, not all data are consistent with this hypothesis. First, activation of p70 S6 kinase...
kinase by some agonists is relatively resistant to wortmannin, suggesting that PI 3-kinase-independent signaling pathways might be involved in activation of the kinase (11). Second, a PDGF receptor mutant (Y740F) that failed to activate PI 3-kinase or Akt activated p70 S6 kinase almost as well as the wild-type receptor (7, 13). Third, although expression of a dominant-negative mutant of Akt-1 in CHO cells inhibited insulin-induced activation of p70 S6 kinase by 75%, the same mutant had only a small inhibitory effect on p70 S6 kinase when expressed in 3T3-L1 adipocytes (18). Finally, Thomas and co-workers (20) have shown that activated mutants of Akt must be constitutively targeted to the membrane in order to stimulate p70 S6 kinase. They have proposed that these mutants artificially induce p70 S6 kinase activation and that results obtained with these mutants may not reflect wild-type Akt signaling (20). Interestingly, membrane-bound versions of active PI 3-kinase were also more efficient than cytosolic mutants at activating p70 S6 kinase (15). When expressed in COS-7 cells, the two forms of PI 3-kinase generated distinct patterns of phosphoinositides (15), raising the possibility that novel phospholipids generated in cells expressing these mutants induce p70 S6 kinase activation.

PI 3,4-bisphosphate and PI 3,4,5-trisphosphate levels in intact cells (Fig. 2B) and PI 3-kinase activity in phosphotyrosine immunoprecipitates (Fig. 4) do not increase in response to PE treatment, thus leading us to conclude that activation of p70 S6 kinase by the α1A-AR is PI 3-kinase-independent. Why then is the activation of p70 S6 kinase by PE inhibited by LY294002 (Fig. 1B)? One possibility is that LY294002 inhibits a protein distinct from PI 3-kinase that is required for p70 S6 kinase activation (15). It has been shown that LY294002 and wortmannin inhibit the in vitro autophosphorylation of the mammalian target of rapamycin (mTOR), a kinase that positively regulates p70 S6 kinase (47). Therefore, the inhibitory effect of LY294002 on p70 S6 kinase activation could be exerted through mTOR.

PKC and Ca^{2+} have been suggested to participate in PI 3-kinase-independent pathways that lead to p70 S6 kinase activation. Similar to the results obtained here with PE (Figs. 1 and 2), exposure of a T cell leukemia line to PMA resulted in activation of p70 S6 kinase with no increase in Akt-1 activity (16). As expected for a PI 3-kinase-independent response, activation of p70 S6 kinase by PMA is constitutively targeted to the membrane in order to stimulate p70 S6 kinase (11, 12). A previous study reported that p70 S6 kinase in HEK293 cells (12). This response was abolished after long term treatment of the cells with PMA, indicating that phospholipase C activates p70 S6 kinase through PKC in this cell type (12). By contrast, we found that down-regulation of PKC in Rat-1 cells by long term PMA treatment has no effect on the activation of p70 S6 kinase by PE (Fig. 1C). Therefore, it appears that PI 3-kinase-independent activation of p70 S6 kinase by the α1A-AR in Rat-1 cells is mediated by a pathway that does not involve PMA-sensitive isoforms of PKC.

It has been known for some time that treatment of cells with A23187 induces the activation of p70 S6 kinase (11, 41), and recent work has demonstrated that Ca^{2+} ionophores and the Ca^{2+}-mobilizing agent thapsigargin activate p70 S6 kinase independently of Akt-1 (35). Consistent with this observation, treatment of cells with these agents causes little or no activation of PI 3-kinase (11, 35). On the other hand, activation of p70 S6 kinase by Ca^{2+} ionophores and thapsigargin is wortmannin-sensitive (11, 35). These cellular responses are similar to those we observed here using PE (Figs. 1, 2, and 4). Thus, activation of p70 S6 kinase by the α1A-AR in Rat-1 cells may be mediated by a Ca^{2+}-dependent pathway (Fig. 6C). Recent reports using EGTA-containing medium or BAPTA-AM to deplete cells of free intracellular Ca^{2+} have indicated that growth factor signaling to p70 S6 kinase is Ca^{2+}-dependent, whereas Akt activation occurs via a Ca^{2+}-independent pathway (35, 48). Our results here (Fig. 6D) and elsewhere (49) confirm and extend these findings. We have found that the PE-induced activation of p70 S6 kinase is greatly reduced in Rat-1 cells depleted of free intracellular Ca^{2+} (49). By contrast, the response of Akt to IGF-1 is normal in Ca^{2+}-depleted cells (Fig. 6D). The Ca^{2+}-dependent step in p70 S6 kinase activation has not yet been identified.

In contrast to its stimulatory effect on p70 S6 kinase (Fig. 1), we found that PE negatively regulates Akt-1 (Fig. 3). The IGF-I-induced activation of Akt was inhibited in cells treated with A23187, suggesting that a high [Ca^{2+}], inhibits signaling to this kinase (Fig. 6A). Surprisingly, although PE induces an increase in [Ca^{2+}], the inhibitory effect of the α1A-AR on Akt activation does not seem to be exerted through a Ca^{2+}-mediated pathway. In Ca^{2+}-depleted cells, PE was still unable to activate Akt, and its inhibitory effect on the IGF-I response was largely intact (Fig. 6B). It is not known whether Akt activity might be inhibited in other physiological settings that involve intracellular Ca^{2+} release.

Consistent with its effect on Akt-1, we also found that treatment of cells with PE reduced the IGF-I-induced PI 3-kinase activation measured in phosphotyrosine immunoprecipitates (Fig. 4). Prior studies have shown that increased serine phosphorylation of the adaptor protein IRS-1 induced by a variety of factors inhibits the ability of the protein to be tyrosine-phosphorylated by the insulin receptor, thus preventing IRS-1 from binding and activating PI 3-kinase (44–46). Two mechanisms have been proposed to mediate the serine phosphorylation of IRS-1. Activators of PKC are thought to promote the phosphorylation of IRS-1 at serine 612 by mitogen-activated protein kinases (44, 45), whereas other factors such as PDGF are thought to regulate IRS-1 function negatively through the phosphorylation of three other serines via an Akt-dependent pathway (45). Inhibition of PI 3-kinase by the α1A-AR does not appear to involve either of these two mechanisms because (a) PE treatment of Rat-1 cells does not activate Akt (Fig. 2A) or the mitogen-activated protein kinases Erk1 and Erk2 (42) and (b) PE also inhibits the activation of PI 3-kinase induced by the PDGF receptor (Fig. 5A), which does not utilize IRS-1 for signaling. Alternative mechanisms to explain the inhibitory effect of the α1A-AR on PI 3-kinase activity might be that it inhibits tyrosine phosphorylation of the p85 subunit of PI 3-kinase or prevents association of p85 with the p110 catalytic subunit of PI 3-kinase. In preliminary experiments, we observed no difference on Western blots in the pattern of tyrosine-phosphorylated proteins from cells treated with or without PE.3 Attempts to examine the p85-p110 interaction in co-immunoprecipitates

3 M. E. Cross and R. Z. Lin, unpublished data.
were unsuccessful due to the low amount of these proteins expressed in Rat-1 cells.\(^3\) We are continuing to investigate the mechanism by which the \(\alpha_{1A}-\text{AR}\) negatively regulates PI 3-kinase.

Our results indicate that the \(\alpha_{1A}-\text{AR}\) differs from the insulin/IGF-I receptor in its ability to activate PI 3-kinase (Figs. 2B and 4). This result may have significant physiologic implications for cell survival. It is well recognized that \(G_{i}\)-coupled and \(G_{q}\)-coupled receptors play an important role in the development and ultimate decompensation of cardiac hypertrophy (50). It was recently demonstrated that overexpression of \(G_{q}\) leads to increased apoptosis of cardiac myocytes both in vitro and in vivo (51). In contrast, treatment with IGF-I stimulates cardiac myocyte hypertrophy but improves cardiac function in experimental models of heart failure (52). This disparity may be explained by the differential activation of PI 3-kinase and Akt by the IGF-I receptor versus the \(\alpha_{1A}-\text{AR}\) (or, possibly, \(G_{q}\)-coupled receptors in general). Perhaps exposure of cardiac myocytes to \(\alpha_{1A}-\text{AR}\) agonists or IGF-I activates p70 S6 kinase, leading to increased protein synthesis and cellular hypertrophy, whereas only IGF-I activates Akt, providing a survival signal. Indeed, our results show that IGF-I treatment has a protective effect against UV-induced apoptosis, whereas PE treatment enhanced the apoptotic effect of UV irradiation (Fig. 7). Studies in rat neonatal cardiac myocytes are currently being done to test the validity of this clinically relevant hypothesis.

To our knowledge, the finding that activation of a \(G_{q}\)-coupled receptor leads to inhibition of Akt and PI 3-kinase has not been previously reported. By using COS-7 cells overexpressing m1 muscarinic acetylcholine receptors and epitope-tagged Akt, Gutkind and co-workers (53) found that activation of this \(G_{q}\)-coupled receptor very weakly stimulated Akt. The difference between finding and the results reported here may be due to cell type differences and alteration in the regulation of Akt when it is overexpressed. Activation of the \(\gamma\) subtype of PI 3-kinase by \(G_{q}\)-coupled receptors via G protein \(\beta\gamma\) subunits is well described in the literature (54, 55). Not surprisingly, recent reports indicated that \(G_{q}\)-coupled receptors also activate Akt (56, 57). Inhibition of Akt by \(\alpha_{1A}-\text{ARs}\) has important physiologic implications for Akt-mediated glucose regulation. Akt is thought to be a critical molecular switch for insulin-mediated regulation of glucose metabolism (23). The possibility that \(G_{q}\)-coupled receptors negatively regulate these insulin-induced effects opens new avenues for research examining the role of this large family of receptors in the development of insulin resistance and diabetes mellitus.

REFERENCES

1. Amaldi, F., and Pierandrei-Amaldi, P. (1997) \textit{Prog. Mol. Subcell. Biol.} 18, 1–17
2. Meyuhas, O., Avni, D., and Silvian, S. (1996) in \textit{Translational Control} (Hershey, J., Mathews, M. B., and Sonenberg, N., eds) pp. 363–388, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Jeffries, H., and Thomas, G. (1996) in \textit{Translational Control} (Hershey, J., Mathews, M. B., and Sonenberg, N., eds) pp. 389–409, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
4. Jeffries, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, B. R., and Thomas, G. (1997) \textit{EMBO J.} 16, 3693–3704
5. Pullen, N., and Thomas, G. (1997) \textit{FEBS Lett.} 410, 78–82
6. Moser, A. B., Dennis, P. B., Pullen, N., Pearson, R. B., Williamson, N. A., Wettenshall, R. E., Kozma, S. C., and Thomas, G. (1997) \textit{Mol. Cell. Biol.} 17, 5648–5655
7. Burgisser, B. M., and Coffer, P. J. (1995) \textit{Nature} 376, 599–602
8. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazalessakas, A., Morrison, D. K., Kabani, K., and Tischla, P. N. (1995) \textit{Cell} 81, 727–736
9. Kohn, A. D., Kozlovski, M. T., and Williams, L. T. (1996) \textit{Mol. Cell. Biol.} 16, 2488–2495
10. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kohn, C. R. (1994) \textit{Mol. Cell. Biol.} 14, 4902–4911
11. Petrich, C., Wascholzki, R., Edelman, H. M., Parker, J. P., and Ballou, L. M. (1995) \textit{Eur. J. Biochem.} 230, 431–438
12. Chung, J., Grammer, T. C., Lemon, K. P., Kazalessakas, A., and Blenis, J. (1994) \textit{Nature} 370, 71–75
13. Ming X. F., Burgering, B. M., Wennstrom, S., Claesson-Welsh, L., Heldin, C. H., Bos, J. L., Kozma, S. C., and Thomas, G. (1994) \textit{Nature} 371, 426–429
14. Didichenko, S. A., Tilton, B., Hemmings, B. A., Ballmer-Hofner, K., and Thelen, M. (1996) \textit{Curr. Biol.} 6, 1271–1278
Differential Regulation of the Phosphatidylinositol 3-Kinase/Akt and p70 S6 Kinase Pathways by the $\alpha_{1A}$-Adrenergic Receptor in Rat-1 Fibroblasts
Lisa M. Ballou, Michael E. Cross, Siqi Huang, E. Michael McReynolds, Bin-Xian Zhang and Richard Z. Lin

*J. Biol. Chem.* 2000, 275:4803-4809.
doi: 10.1074/jbc.275.7.4803

Access the most updated version of this article at [http://www.jbc.org/content/275/7/4803](http://www.jbc.org/content/275/7/4803)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 55 references, 33 of which can be accessed free at [http://www.jbc.org/content/275/7/4803.full.html#ref-list-1](http://www.jbc.org/content/275/7/4803.full.html#ref-list-1)