Akt Mediates Sequestration of the β2-Adrenergic Receptor in Response to Insulin*

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Received for publication, September 11, 2001, and in revised form, December 20, 2001
Published, JBC Papers in Press, January 24, 2002, DOI 10.1074/jbc.M108771200

The counterregulation of catecholamine action by insulin includes insulin-stimulated sequestration of the β2-adrenergic receptor. Herein we examined the signaling downstream of insulin receptor activation, focusing upon the role of 1-phosphatidylinositol 3-kinase and the serine-threonine protein kinase Akt (also known as protein kinase B) in the internalization of β2-adrenergic receptors. Inhibition of 1-phosphatidylinositol 3-kinase by LY294002 blocks insulin-induced sequestration of the β2-adrenergic receptor, implicating Akt in downstream signaling to the β2-adrenergic receptor. Phosphorylation studies of the C-terminal cytoplasmic domain of the β2-adrenergic receptor by Akt in vitro identified Ser345 and Ser346 within a consensus motif for Akt phosphorylation. Double mutation (i.e. S345A/S346A) within this motif abolishes insulin counterregulation of β2-adrenergic stimulation of cyclic AMP accumulation as well as insulin-stimulated sequestration. Furthermore, expression of constitutively activated Akt (T308D/S473D) mimics insulin action on cyclic AMP responses and β2-adrenergic receptor internalization. Expression of the dominant-negative version of Akt (K179A/T308A/S473A), in contrast, abolishes both insulin counterregulation of the cyclic AMP response as well as insulin-stimulated sequestration of the β2-adrenergic receptor. The action of the serine-threonine protein kinase Akt in insulin counterregulation mirrors the central role of protein kinase A in β-adrenergic-induced desensitization.

Protein phosphorylation plays a prominent role in growth factor signaling (1, 2). It is well recognized that the activation of 1-phosphatidylinositol 3-kinase (PI3-kinase) and its downstream protein kinase cascade is an essential and early event in signaling by insulin (3). PI3-kinase has been implicated in diverse cellular functions, including mitogenesis, growth factor receptor regulation, actin-cytoskeleton rearrangement, and intracellular trafficking of proteins (4). The products generated by the PI3-kinase reaction activate 3-phosphoinositide-dependent kinase (PDK1) (5), which in turn, activates a serine-threonine protein kinase Akt. Akt has been shown to be a key element in insulin signaling downstream of PI3-kinase and in the trafficking of the insulin-sensitive transporter GLUT4 (6, 7). Mice lacking Akt2, for example, display insulin resistance and a diabetes mellitus-like state (8), reflecting interruption of insulin signaling to GLUT4 trafficking as well as to other downstream signaling.

Insulin counterregulates catecholamine action, a facet of insulin action that includes insulin-stimulated phosphorylation and trafficking of β2-adrenergic receptors (β2AR) (9–14). Sequestration of β2AR occurs in response to counterregulation by insulin and also in response to chronic stimulation of β2AR by β-adrenergic agonist, a late phase of agonist-induced desensitization (15, 16). For agonist-induced desensitization, activation of G-protein-coupled receptor kinases (GRK) and protein kinase A play critical roles in catalyzing phosphorylation of the β2AR required for eventual internalization (15). As counterregulation of β2AR by insulin involves changes in phosphorylation and the trafficking of the receptor, we were intrigued by the possibility that Akt may be mediating these effects of insulin on this well known member of the superfamily of G-protein-coupled receptors (GPCRs). In the current work, we investigate the nature of the signaling downstream of insulin activation of PI3-kinase, focusing upon the role of Akt. We show that activation of PI3-kinase and Akt is essential for insulin counterregulation and sequestration of β2AR.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Human epidermoid carcinoma (A431) and Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagles medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT), plus penicillin (60 μg/ml) and streptomycin (100 μg/ml), grown in a humidified atmosphere of 5% CO2 and 95% air at 37 °C (17). For epifluorescence imaging, the larger A431 cells proved to be superior for localization studies to the CHO cells, and so this analysis was confined to the A431 cells.

Transfection of GFP-tagged β2-Adrenergic Receptor—Cells were transfected with expression vectors harboring the GFP-tagged β2AR using LipofectAMINE® (Invitrogen), according to the manufacturer’s protocol, and viable clones were selected in 400 μg/ml of the neomycin analog G418. Resistant colonies were subcloned and screened for GFP fusion protein expression by epifluorescence microscopy. Expression of β2AR was monitored by radioligand binding, using iodocyanopindolol (ICYP) as the labeled probe (18). Cell lysates and crude membranes were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels, transferred electrophoretically to nitrocellulose blots, and the blots stained with specific antibodies, as reported earlier. The antibodies employed in these studies and their suppliers were as follows: anti-phosphotyrosine-pY69 (anti-pY) mouse monoclonal (Transduction Laboratories); anti-phosphoserine (anti-pS) rabbit polyclonal (Zymed Laboratories); anti-phosphothreonine (anti-pT) rabbit polyclonal (Zymed Laboratories Inc.); anti-β2AR-CM04 (Malbon Labora-
LY294002 compound inhibits insulin-stimulated β2AR desensitization. Time course of isoproterenol-stimulated cyclic AMP accumulation without (panel A) or with 10 μM LY294002 (panel C), or both insulin and LY294002 (panel D) in CHO-K1 cells stably expressing β2AR. Cells were pretreated with insulin for 15 min and then challenged with isoproterenol (10 μM) concurrently for 5 min. To investigate the possible role of PI3-kinase in these responses, cells were exposed to 10 μM LY294002 compound for 60 min prior to the addition of insulin. The time course for insulin-stimulated counterregulation of isoproterenol-stimulated cyclic AMP accumulation is shown (panel F). Inhibitor Studies—Stably transfected clones were routinely challenged in cell medium following serum deprivation for 8–12 h without or with 100 nM insulin for 15 min and the trafficking of the GFP-tagged β2AR monitored by epifluorescence microscopy. Cells were serum-deprived for 8 h prior to removing growth factors and catecholamines from the cell medium. PI3-kinase was inhibited using the LY294002 compound (10 μM). For studies of the effects of inhibitor on the trafficking of the GFP-tagged receptor in response to insulin, the inhibitor was added 30 min in advance of the challenge with insulin.

Phosphorylation of the C-terminal Cytoplasmic Domain of the β2AR by Akt—The fusion protein of GST with the C-terminal, cytoplasmic domain of β2AR (BAC1-C1 protein) was phosphorylated at 37 °C in a 50-μl reaction mixture containing 0.05 Tris-HCl, 0.1% Nonidet P-40, 0.15 M NaCl, 5 mM dithiothreitol, 5 mM MgCl2, 0.1 mM γ-32P][ATP (0.5–2.0 × 106 cpm/mol), 0.02 unit/μl Akt (Upstate Biotechnology) and BAC1 (1.0 μg). One unit of Akt activity corresponds to 1 pmol of phosphate transfer per min at pH 7.4 at 30 °C. Phosphorylation was initiated by the addition of the enzyme to the reaction mixture. After a 30-min incubation, the reaction mixture was treated with 0.1% SDS and 50 mM dithiothreitol for 5 min at 75 °C and subjected to SDS-polyacrylamide gel electrophoresis. The resolved protein gels were fixed in 10% acetic acid, stained, and the radiolabeled proteins made visible by autoradiography.

Assay of Intracellular Accumulation of Cyclic AMP and Counterregulation by Insulin—For assay of cyclic AMP accumulation, stably transfected A431 cells were seeded in 24-well plates 48 h prior to determination, at a density of 1 × 105 cells/well. On the day of experiment, cell culture medium was aspirated, the cells washed and replenished with Krebs-Ringer phosphate medium containing 10 μM RO-201724 (cyclic AMP phosphodiesterase inhibitor), and then treated with the isoproterenol (10 μM) for 5 min in a total assay volume of 300 μl. The reaction was terminated by the addition of 100 μl of 100% ethanol, and the cyclic
AMP content measured by the competitive binding assay, as described (11). To assay insulin counterregulation, cells were pretreated with or without LY294002 for 30 min and then challenged with 100 nM insulin for 15 min. HA-β2AR was isolated from cell lysate by immunoprecipitation with HA-specific antibodies, subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose blots, and stained with antibodies specific for phosphoserine (pS), phosphothreonine (pT), phosphotyrosine (pY) or β2AR (CM-2). The results displayed are sample images, representative of more than four separate experiments. Panel B, in vitro phosphorylation of a GST fusion protein with the C-terminal, cytoplasmic domain of β2AR (C1). GST fusion proteins with progressively truncated C-terminal cytoplasmic domains of β2AR (C4-C6) or the GST itself were incubated with recombinant, activated Akt. The labeled products were separated in 10% SDS-polyacrylamide gels and visualized by autoradiography. Protein constructs included the full-length C-terminal domain of β2AR (C1, Arg328-Leu413 fragment), Arg328-Tyr366 fragment (C4), Arg328-Asn352 fragment (C5), Arg328-Cys341 fragment (C6) and GST itself. Arrows show positions of C-terminal domain phosphorylation by Akt, as deduced by the studies. The results displayed are sample images, representative of more than four separate experiments. Panels C and D, mass spectrometry of tryptic digests of β2AR obtained from A431 cells that were untreated or treated with 100 nM insulin for 15 min. The HA-tagged β2AR were isolated from the cells and subjected to tryptic digestion (9,10), followed by analysis of the peptides on a ABI voyager research-grade MALDI mass spectrometer operated in the linear mode (panel C) or on the linear versus reflectron mode (panel D).

MALDI Time-of-Flight Mass Spectrometry for Analysis of Receptor Phosphorylation—Confluent cultures of A431 cells were serum-starved for 12 h and then treated without and with 100 nM insulin for 15 min. In each case, cultures were pooled from five 100 mm Petri dishes. The
cells were lysed and the β2AR subjected to immunoprecipitation with antibody CM04, using 25 μg of antibody per 0.1 ml of A/G agarose. Immunoprecipitates were digested with 1 μg of trypsin for 8 h at room temperature. The phosphate-containing peptides were isolated on Fe³⁺ columns (0.1 ml volume) and then analyzed on an ABI Voyager DE-STR mass spectrometer using α-cyano-4-hydroxycinnamic acid as the matrix.

RESULTS AND DISCUSSION

We measured the ability of insulin to counterregulate β-adrenergic-stimulated cyclic AMP in CHO clones expressing ~30,000 β-adrenergic receptors per cell (Fig. 1). Isoproterenol (10 μM) stimulated rapid accumulation of intracellular cyclic AMP (Fig. 1A). Treatment with insulin (100 nM) 15 min in advance of challenge with the β-adrenergic agonist inhibits the cyclic AMP response of the cells to stimulation by the β-adrenergic agonist isoproterenol, reducing the response by 50% (Fig. 1B). PI3-kinase has been shown to be an obligate, early downstream element in insulin signaling (3). We probed the possible role of PI3-kinase in insulin counterregulation of catecholamine-stimulated cyclic AMP accumulation using the PI3-kinase inhibitor LY294002. Treatment with LY294002 (10 μM) alone had little effect on β-adrenergic stimulation of intracellular cyclic AMP accumulation (Fig. 1C); whereas it abolished the counterregulatory effect of insulin on cyclic AMP accumulation in response to isoproterenol (Fig. 1D), suggesting that the counterregulatory effects of insulin on catecholamine action share PI3-kinase activation upstream. The time course for insulin-stimulated counterregulation of β2AR was rapid, demonstrable intracellular accumulation (yellow arrowheads) and cell membrane thinning (white arrows) of β2AR observed within minutes of stimulation by insulin (Fig. 2A). Insulin-stimulated counterregulation of β2AR was examined in the absence and the presence of the LY294002 inhibitor (Fig. 2B). The A431 cells display the same counterregulatory effects of insulin on β2AR signaling as was observed in the CHO clones. Epifluorescence studies of GFP-tagged β2AR in A431 cells revealed that in the presence of insulin, β2ARs underwent a prominent sequestration, internalizing from the cell membrane (white arrows) into perinuclear regions of the cells (Fig. 2C, yellow arrowheads). The magnitude of the insulin-stimulated sequestration reaches ~60% of the cellular complement of β2AR and is of greater magnitude than that stimulated by β-adrenergic agonist-induced sequestration (14). Treating the cells with LY294002, which abolishes the ability of insulin to inhibit catecholamine-stimulated cyclic AMP response, also abolished the ability of insulin to provoke the internalization of β2AR (Figs. 1F and 2B). Treatment with the LY294002 compound alone produced no significant effect on the cyclic AMP response (Figs. 1F and 2B) or on the localization of β2AR (Fig. 2C).

Because activation of PI3-kinase results in the activation of
Akt resulted in increased phosphorylation of the HA-tagged version of the human wild-type or S345A/S346A double mutant (AA mutant) lacking the Akt phosphorylation motif and the cells challenged with 0.1 μM insulin for 15 min. Receptor phosphorylation was assayed following receptor immunoprecipitation from whole cell lysates using HA-specific antibodies. The immunoprecipitates were subjected to immunoblotting and stained with antibodies against phosphoserine (pS), phosphothreonine (pT), or phosphotyrosine (pY), as described in legend for Fig. 2. The results displayed are sample images, representative of more than four separate experiments.

downstream serine-threonine protein kinases, like Akt, we examined the phosphorylation state of the β2AR (Fig. 3). An HA-tagged version of the human β2AR was expressed in CHO cells, and the cells were treated with insulin (Fig. 3A). Treatment with insulin (100 nM) resulted in increased phosphorylation of the β2AR, as detected with phosphoserine-, phosphothreonine-, and phosphotyrosine-specific antibodies. The β2AR has been shown both in vitro and in vivo to be a substrate for tyrosine phosphorylation catalyzed by the insulin receptor itself (9), confirmed herein (Fig. 3A) (10, 11). Increases in phosphoserine and phosphothreonine content of the β2AR in response to insulin had been noted earlier (9), but the protein kinase(s) responsible for this phosphorylation remained elusive. To test further the nature of the serine phosphorylation of the β2AR, we investigated the effects of expression of a constitutively activated mutant of Akt (T308D/S473D; CA-Akt) on the phosphorylation state of the β2AR. Co-expression of CA-Akt resulted in increased phosphorylation of β2AR that mimicked that observed in cells stimulated by insulin (Fig. 3A). Thus, stimulation of cells with insulin or expression of CA-Akt leads to increased phosphoserine content of the β2AR.

The desensitization and sequestration of β2AR in response to the β-adrenergic agonist is mediated largely by phosphorylation of the receptor on its C-terminal, cytoplasmic domain (15, 16). We hypothesized that Akt might be playing a role similar to GRK and/or protein kinase A on this domain downstream of insulin activation of PI3-kinase. Akt is a serine-threonine-specific protein kinase activated in response to the activation of PI3-kinase, so we examined the β2AR sequence for a motif found in other Akt substrates (21, 22). Embedded in the C-terminal, cytoplasmic domain of the β2AR is a consensus site for Akt phosphorylation (21), namely Cys141→Asn152 that contains RRSSLKAY (Fig. 3B).

We tested this hypothesis in vitro by preparing a series of GST-tagged proteins corresponding to the C-terminal domain of the β2AR progressively truncated from the C terminus (23). The full-length, C-terminal domain of the β2AR (C1) has been shown to be readily phosphorylated by protein kinase A, GRK2, and the insulin receptor tyrosine kinase (24). We used this in vitro approach with purified, activated Akt to establish the sites of β2AR phosphorylation and test if the canonical motif for Akt was, in fact, a substrate (Fig. 3B). The peptide corresponding to the full-length, C-terminal cytoplasmic domain (GST-Arg130→Leu413) of the β2AR was a substrate for Akt-catalyzed phosphorylation. Deletion mutants of the C-terminal domain of the β2AR were prepared (C4, C5, and C6) and employed in the phosphorylation reaction. Peptides C4 (Arg130→Tyr366) and C5 (Arg130→Asn392), like the C1 protein itself, were phosphorylated by Akt. Deletion of Leu342→Leu413 (C6) of the β2AR C-terminal domain, in contrast, resulted in the loss of phosphorylation by Akt. These results provide evidence that the RRSSLKAY motif in the C-terminal domain of the β2AR is phosphorylated by Akt. The phosphoamino acid analysis performed by immunoblotting (Fig. 3A), likewise confirms the results that Akt phosphorylates serine residues of the β2AR.

Having established the ability of the β2AR to act as a substrate for phosphorylation by Akt in vitro, we used this information to ascertain the phosphorylation of this site of the β2AR in vivo. To determine whether the canonical site for Akt phosphorylation indeed was phosphorylated in response to insulin, we stimulated A431 cells with insulin, isolated the β2AR, and determined the phosphorylation state of the receptor peptide fragment that harbors the canonical site. β2AR from A431 cells treated with and without insulin (100 nM, 30 min) were isolated, digested with trypsin, and subject to MALDI mass spec-
trometry (Fig. 3C). The analysis identified the phosphorylated peptide SSLK (495.6 m/z, denoted by asterisk). The amount of the phosphorylated peptide was increased in tryptic digests from β2AR isolated from the insulin-treated as compared with untreated cells (Fig. 3C). We employed mass spectrometry to test if the mass change was due to phosphorylation by comparing the spectrum of the mass analysis performed in the linear compared with the reflectron mode. Serine-phosphorylated peptides are easily lost to in-source (25) and post-source (26) decay. This behavior is diagnostic for serine-phosphorylated peptides are easily lost to in-source (25) and post-source (26) decay. This behavior is diagnostic for serine-phosphorylated peptides, as denoted by asterisk.

Akt and Internalization of β2AR

It was important to ascertain if the Ser345, Ser346 residues of the β2AR were critical to the ability of insulin to counterregulate the cyclic AMP response of the cells to isoproterenol stimulation. We prepared the S345A/S346A (AA) double substitutions in the β2AR and examined the ability of insulin to inhibit the cyclic AMP response and induce β2AR sequestration (Fig. 4). The S345A/S346A double mutant β2AR no longer demonstrates sensitivity to insulin with respect to isoproterenol-stimulated cyclic AMP accumulation (Fig. 4A). The AA mutant β2AR displayed normal activation in response to isoproterenol (data not shown). Similarly, the AA mutant β2AR was not sequestered in response to insulin (Fig. 4B). The sequestration observed in response to insulin stimulation for β2AR (Figs. 2A and 4B) was tested for another member of the superfamily of G-protein-coupled receptors, the 5HT2a serotonin receptor. The 5HT2a receptor was not sequestered either by 100 nM insulin (+Ins) stimulation or stimulation with the β2AR agonist, isoproterenol (Fig. 4C, +Ins). Thus, the β2AR receptor is selectively sequestered in response to insulin stimulation, and the serine residues that can act as substrates for Akt-catalyzed phosphorylation are essential for the counterregulatory effects of insulin on the β2AR.
We gathered additional data on the phosphorylation of Ser\(^{345}\), Ser\(^{346}\) sites of the \(\beta 2\)-AR by study of the receptors in vivo. Cells expressing the wild-type (WT) and the S345A/S346A (AA) double mutant form of the \(\beta 2\)-ARs were treated with insulin (100 nM), and the phosphorylation state of the receptors was established through immunoblotting with phosphoamino acid-specific antibodies, as outlined above. Whereas the wild-type receptor displayed increased protein phosphorylation, the AA mutant failed to show increased phosphoserine content in response to insulin (Fig. 5). Surprisingly, phosphotyrosine content of the AA double mutant receptor did not increase in response to insulin. These data suggest the possibility that the phosphorylation of the Ser\(^{345}\), Ser\(^{346}\) sites and the Tyr\(^{350}\) site may be hierarchical, linked in a manner whereby the phosphorylation of the Ser\(^{345}\), Ser\(^{346}\) sites influences phosphorylation of the Tyr\(^{350}\) residue.

We further probed whether Akt mediates the action of insulin on the counterregulation of the \(\beta 2\)-AR through the use of a constitutively active form of Akt (T308D/S473D; CA-Akt). If Akt is a mediator of insulin action in this signaling pathway, we might expect that activation of Akt would be insulinomimetic (Fig. 6). Immunoblotting of cell extracts from CHO and A431 clones transfected with empty vector or the CA-Akt mutant revealed enhanced immunoreactivity for the clones expressing a mutant form of Akt (Fig. 6A). Expression of the CA-Akt in these cells provoked a reduction in isoproterenol-stimulated cyclic AMP accumulation, much like insulin treatment (Fig. 6B). The addition of insulin (100 nM) to the cells expressing the CA-Akt produced no further reduction in the isoproterenol-stimulated cyclic AMP response. Complementary studies of \(\beta 2\)-AR localization revealed marked internalization of \(\beta 2\)-AR in cells expressing the CA-Akt, resembling the situation noted when the cells were treated with insulin. Addition of insulin failed to alter significantly the marked internalization of \(\beta 2\)-AR observed in the cells expressing CA-Akt (Fig. 6C). These data suggest that with respect to the counterregulatory effects of insulin on \(\beta 2\)-AR action, CA-Akt is fully insulinomimetic.

We made use of a dominant-negative strategy to test further the role of Akt on the counterregulatory influence of insulin on \(\beta 2\)-AR (Fig. 7). The mutant form (K179A/T308A/S473A) of Akt (DN-Akt) was expressed in the cells and the ability of insulin to inhibit \(\beta\)-adrenergic stimulation of cyclic AMP accumulation (Fig. 7A) and \(\beta 2\)-AR sequestration (Fig. 7B) measured. Expression of DN-Akt itself resulted in a small reduction in the cyclic AMP response to isoproterenol (Fig. 6A). For cells expressing the DN-Akt, the ability of insulin to counterregulate the \(\beta\)-adrenergic cyclic AMP response was abolished. Moreover, in the cells expressing DN-Akt, not only was the counterregulation by insulin abolished, but also treatment with insulin produced a significant increase in the cyclic AMP response. Analysis of \(\beta 2\)-AR localization in cells expressing DN-Akt revealed two novel features (Fig. 7B). In comparison with the control cells, the cells expressing DN-Akt displayed somewhat more intracellular GFP-tagged \(\beta 2\)-AR (arrowheads) in the unstimulated state. This observation is not at all unexpected, because Akt is known to influence the trafficking of membrane proteins, such as GLUT4 glucose transporter. The DN-Akt may well suppress the counter movement of \(\beta 2\)-AR to the cell membrane from intracellular compartment. Surface GFP-tagged \(\beta 2\)-AR were also readily observed (arrows) in the cells expressing DN-Akt, but unlike the control cells, these cells did not display marked sequestration of \(\beta 2\)-AR from the cell membrane in response to insulin. Thus, expression of DN-Akt abolished the ability of insulin to counterregulate the \(\beta\)-adrenergic-stimulated cyclic AMP response as well as the ability of insulin to internalize \(\beta 2\)-AR.

The current studies illuminate a novel role of Akt mediating insulin counteraction of catecholamine action. Earlier observations demonstrated the following: insulin stimulates inactivation of the \(\beta 2\)-AR through tyrosyl phosphorylation (10); phosphorylation of \(\beta 2\)-AR Tyr\(^{350}\) in response to insulin creates a SH2 binding site with which Grb2, dynamin, and the p85 subunit of PI3-kinase can interact (9); and insulin stimulates marked sequestration of the \(\beta 2\)-AR, which requires the integrity of Tyr\(^{350}\) and activation of PI3-kinase (14). Herein we show that Akt plays an obligate role for insulin signaling to the \(\beta 2\)-AR. Elimination of an Akt phosphorylation motif renders the \(\beta 2\)-AR insensitive to counterregulation by insulin and unable to be sequestered in response to insulin. Expression of dominant-negative Akt blocks the ability of insulin to sequester \(\beta 2\)-AR, whereas expression of a constitutively active Akt mimics the effects of insulin on \(\beta 2\)-AR sequestration. Akt is well known to regulate endosomal trafficking, acting in concert with EEA1 and Rab5 (3). Akt activation results in a shuttling of the insulin-sensitive GLUT4 transporter-laden vesicles to the cell membrane. In a similar manner, Akt may be causing shuttling of newly formed, phospho-\(\beta 2\)-AR-laden vesicles to intracellular locales. For agonist-stimulated sequestration of \(\beta 2\)-AR, both the serine-threonine kinases, protein kinase A and GRK, function to traffic receptors from the cell membrane to endosomes for re-cycling or degradation (15). For insulin-stimulated sequestration, Akt appears to play an analogous role trafficking \(\beta 2\)-AR to intracellular locales (Fig. 8). Insulin stimulates its receptor to autophosphorylate, phosphorylate the \(\beta 2\)-AR at Tyr\(^{350}\), Tyr\(^{364}\), and Tyr\(^{366}\), as well as IRS1,2, to thereby activate PI3-kinase activity. Activation of PI3-kinase leads to activation of PDK1 and downstream to activation of Akt. Akt, in turn, phosphorylates the \(\beta 2\)-AR on an Akt canonical site located in the cytoplasmic, C-terminal tail and provokes the sequestration and internalization of the \(\beta 2\)-AR. The interplay between PI3-kinase, Akt, and endosomal elements with the \(\beta 2\)-AR seems to be quite complex and will require further understanding of the intracellular trafficking pathways.

Acknowledgments—We thank Dr. Philip Cohen (Department of Biochemistry, University of Dundee, Scotland, UK) for providing to us the dominant-negative and constitutively active forms of Akt2.

REFERENCES

1. Schlessinger, J. (2000) Cell 103, 211–225
2. Hunter, T. (2000) Cell 100, 113–127
3. Corvera, S., and Czech, M. P. (1998) Trends Cell Biol. 8, 442–446
4. Alessi, D. R., and Cohen, P. (1998) Curr. Opin. Genet. Dev. 8, 55–62
5. Alessi, D. R. (2001) Biochem. Soc. Trans. 29, 1–14
6. Summers, S. A., Whiteman, E. L., and Birnbaum, M. J. (2000) Int. J. Obs. Relat. Metab. Disord. 24, Suppl. 4, S67–S70
7. Olefsky, J. M. (1999) J. Biol. Chem. 274, 1863
8. Cun, H., Mu, J., Kim, J. K., Thorvaldsen, J. L., Chu, Q., Crenshaw, E. B., III, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001) Science 292, 1728–1731
9. Baltensperger, K., Karoor, V., Paul, H., Russo, A., Czech, M. P., and Malbon, C. C. (1996) J. Biol. Chem. 271, 1061–1064
10. Karoor, V., Baltensperger, K., Paul, H., Czech, M. P., and Malbon, C. C. (1995) J. Biol. Chem. 270, 25305–25313
11. Karoor, V., and Malbon, C. C. (1996) J. Biol. Chem. 271, 29347–29352
12. Karoor, V., and Malbon, C. C. (1998) Adv. Pharmacol. 42, 425–428
13. Karoor, V., Shih, M., Tholainikunnel, B., and Malbon, C. C. (1996) Prog. Neurobiol. 48, 555–568
14. Karoor, V., Wang, L., Wang, H. Y., and Malbon, C. C. (1998) J. Biol. Chem. 273, 33035–33041
15. Morris, A. J., and Malbon, C. C. (1999) Physiol. Rev. 79, 1373–1430
16. Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 18677–18680
17. Wang, H. Y., Berrios, M., and Malbon, C. C. (1999) Biochem. J. 363, 519–532
18. Malbon, C. C. (1980) J. Biol. Chem. 255, 8692–8699
19. Lin, F., Wang, H., and Malbon, C. C. (2000) J. Biol. Chem. 275, 19025–19034
20. Kashles, O., and Levitzki, A. (1987) Biochem. Pharmacol. 36, 1513–1538
21. Ohata, T., Yaffe, M. B., Leparc, G. G., Piro, E. T., Maegawa, H., Kashwagi, A., Kikkawa, R., and Cantley, L. C. (2000) J. Biol. Chem. 275, 36108–36115
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22. Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) *FEBS Lett.* **399**, 333–338
23. Fan, G., Shumay, E., Wang, H. H., and Malbon, C. C. (2001) *J. Biol. Chem.* **276**, 13240–13247
24. Doronin, S., Lin, F., Wang, H., and Malbon, C. C. (2000) *Protein Expr. Purif.* **20**, 451–461
25. Lennon, J. J., and Walsh, K. A. (1999) *Protein Sci.* **8**, 2487–2493
26. Annan, R. S., and Carr, S. A. (1997) *J. Protein Chem.* **16**, 391–402
27. Mann, M., Hendrickson, R. C., and Pandey, A. (2001) *Annu. Rev. Biochem.* **70**, 437–473
28. Steen, H., Kuster, B., and Mann, M. (2001) *J. Mass Spectrom.* **36**, 782–790
29. McLachlin, D. T., and Chait, B. T. (2001) *Curr. Opin. Chem. Biol.* **5**, 591–602