Insulin Stimulates Sequestration of β-Adrenergic Receptors and Enhanced Association of β-Adrenergic Receptors with Grb2 via Tyrosine 350*

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G-protein-linked receptors, such as the β2-adrenergic receptor, are substrates for growth factor receptors with intrinsic tyrosine kinase activity (Karoor, V., Baltensperger, K., Paul, H., Czech, M. P., and Malbon C. C. (1995) J. Biol. Chem. 270, 25305–25308). In the present work, the counter-regulatory action of insulin on catecholamine action is shown to stimulate enhanced sequestration of β2-adrenergic receptors in either DDT, MF-2 smooth muscle cells or Chinese hamster ovary cells stably expressing β2-adrenergic receptors. Both insulin and insulin-like growth factor-1 stimulate internalization of β-adrenergic receptors, contributing to the counter-regulatory effects of these growth factors on catecholamine action. In combination with β-adrenergic agonists, insulin stimulates internalization of 50–60% of the complement of β-adrenergic receptors. Insulin administration in vitro and in vivo stimulates phosphorylation of Tyr-350 of the β-adrenergic receptor, creating an Src homology 2 domain available for binding of the adaptor molecule Grb2. The association of Grb2 with β-adrenergic receptors was established using antibodies to Grb2 as well as a Grb2-glutathione S-transferase fusion protein. Insulin treatment of cells provokes binding of Grb2 to β2-adrenergic receptors. Insulin also stimulates association of phosphatidylinositol 3-kinase and dynamin, via the Src homology 3 domain of Grb2. Both these interactions as well as internalization of the β2-adrenergic receptor are shown to be enhanced by insulin, β-agonist, or both. The Tyr-350 → Phe mutant form of the β2-adrenergic receptor, lacking the site for tyrosine phosphorylation, fails to bind Grb2 in response to insulin, fails to display internalization of β2-adrenergic receptor in response to insulin, and is no longer subject to the counter-regulatory effects of insulin on cyclic AMP accumulation. These data are the first to demonstrate the ability of a growth factor insulin to counter-regulate G-protein-linked receptor, the β-adrenergic receptor, via a new mechanism, i.e. internalization.

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The abbreviations used are: GPLR, G-protein-linked receptor; TKR, growth factor receptor with intrinsic tyrosine kinase activity; β2AR, β2-adrenergic receptor; PI 3-kinase, phosphatidylinositol 3-kinase; SH, Src homology; DMEM, Dulbecco's modified Eagle's medium; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; IGF, insulin-like growth factor; CHO, Chinese hamster ovary; GST, glutathione S-transferase.

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position 350 of the β2AR. Furthermore, insulin is shown to enhance β-adrenergic agonist-induced internalization of β2ARs in a manner that is sensitive to inhibition of PI 3-kinase or to substitution of phenylalanine for tyrosine at position 350 of the receptor (Y350F).

EXPERIMENTAL PROCEDURES

Materials—DDT, MF-2 hamster was defers smooth muscle cells and Chinese hamster ovary (CHO) cells stably expressing wild-type and mutant β2ARs (4, 5) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum. Recombinant insulin receptors were purified from CHO-T cells overexpressing the insulin receptor by affinity chromatography on wheat germ agglutinin coupled to agarose, as described (24). CH-Sepharose was obtained from Amersham Pharmacia Biotech. Antibodies to PI 3-kinase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and those to Grb2 and dynamin from Transduction Labs (Lexington, KY). The GST-Grb2 construct was a gift from Dr. James Bliska (SUNY, Stony Brook, NY) and the fusion protein was expressed in E. coli and purified using standard protocols. The matrix-immobilized GST-Grb2 Sepharose was purchased from Amersham Pharmacia Biotech. Phosphatidylinositol bisphosphate was a kind gift from Dr. Andrew Morris (Department of Pharmacology, SUNY, Stony Brook, NY). Antipeptide antibodies to the hamster β2-adrenergic receptor were either developed in the laboratory (CM-04; Refs. 3–5) or obtained from a commercial supplier (Santa Cruz Biotechnology) and employed throughout these studies.

Co-immunoprecipitation and Immunoblotting—Confluent cultures of DDT, MF-2 cells were serum-starved for 18 h prior to each experiment. Cells were treated with hormones in serum-free DMEM for the indicated times and then lysed in a buffer containing 20 mm Tris-HCl (pH 7.5), 150 mm NaCl, 100 μM sodium vanadate, 40 mM NaF, 1% Nonidet P-40, 1 mM CaCl2, 1 mM MgCl2, 10% glycerol, 20 μM phenylarsine oxide, and a mixture of protease inhibitors (50 μg/ml leupeptin, 50 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml bacitracin, and 100 μg/ml benzamidine). β2AR was immunoprecipitated with antipeptide antibody CM-04 for 5 h at 4 °C (3–5). The CM-04 antibody was generated to a synthetic peptide corresponding to amino acids 99–110 located in the extracellular domain of the β2-adrenergic receptor (3–5), recognizing the Y350F mutant β2-adrenergic receptor as it does the wild-type counterpart (data not shown). In the case of the GST-Grb2 Sepharose beads, aliquots of whole-cell lysates were incubated with 20 μl of the beads for 2 h at 4 °C. The beads were washed exhaustively and the adsorbed proteins solubilized and subjected to SDS-PAGE (4, 5) as described by the commercial supplier. The immunoprecipitates were collected by centrifugation and subjected to SDS-PAGE (4, 5). The resolved proteins were transferred onto nitrocellulose blots, probed with the antibodies indicated, and stained with a secondary antibody to which horseradish peroxidase was coupled. The Western blot chemiluminescence reagent (NEL Life Science Products) and autoradiography were used to detect the immunocomplexes, following the instruction protocols of the supplier. Immunoprecipitates and GST-Grb2 treatments were performed with equivalence in the amount of cell extract protein and amount of β2-adrenergic receptor.

Assay of PI 3-Kinase Activity—PI 3-kinase activity was measured directly, essentially as described (25). The immunoprecipitates were washed twice with phosphate-buffered saline containing 1% Nonidet P-40, and 100 μM sodium vanadate, twice with 100 mM Tris (pH 7.5) containing 0.5 mM LiCl, and twice with the assay buffer (10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100 μM sodium vanadate). The kinase assay was performed directly on beads in a total reaction volume of 50 μl containing 10 μg of immunoprecipitate, 125 μl of HCl, and the cyclic AMP labeled with 32P-ATP) at 30 °C for 5.0 min. The reaction was stopped by the addition of 50 μl of 0.1 M HCl, and the tubes were transferred to an ice slurry. Lipids were then extracted with the addition of 375 μl of chloroform:methanol:HC1 (200:400:5) first, 125 μl of HCl, and 125 μl of chloroform. The tubes were vortexed, and the lower phase was reextracted with synthetic upper phase chloroform:methanol:0.1 M HCl (1:1:1). The lipids were dried under nitrogen and separated by TLC on oxalate-coated silica gel plates in a solvent system containing chloroform:methanol:acetic acid:water (140:200:30:50).

Assay of Intracellular Accumulation of Cyclic AMP—For assay of cyclic AMP accumulation, cells were seeded in 96-well plates 48 h prior to determination, at a density of 1 x 10^6 cells/well. On the day of experiment, medium was aspirated, the cells washed and replenished with Krebs-Ringer phosphate medium containing 10 mM RO-201724 (cyclic AMP phosphodiesterase inhibitor), and then treated with the indicated hormones in a total assay volume of 50 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 (26).

Internalization of β2AR—Radioligand binding was performed on whole cells, as described (27). Cultures of DDT, MF-2 cells were treated with indicated hormones in either the presence or the absence of the PI 3-kinase inhibitor wortmannin (100 nM) at 25 °C for 5 min. The cells were then washed with ice-cold phosphate-buffered saline and resuspended in DMEM containing 20 mM HEPES (pH 7.4) and the hydrophilic, membrane-impermeant β-adrenergic antagonist [HICGP-12177 (70 nM). Binding was performed at 4 °C for 6 h. The cells were diluted, collected on GF/C membranes at reduced pressure, and washed rapidly. The radioligand bound to the washed cell mass on the filter was counted by liquid scintillation spectrometry. Nonspecific binding was defined as the radioligand binding insensitive to competition by the unlabeled, β-adrenergic antagonist propanolol (10 μM), as reported earlier (27, 28). Assay of β2-adrenergic receptors by binding of iodocyanopindolol to intact cells was performed as described (28).

RESULTS

Insulin stimulates phosphorylation of β2ARs on specific tyrosyl residues and attenuates the ability of the receptor to activate Gs in response to β-adrenergic agonist (3–5). PI 3-kinase activation is critical to intracellular trafficking of membrane-bound receptors (14, 15) and is an early event in insulin action (16–18). We explored to what extent, if any, does internalization of β2ARs represent a counter-regulatory effect of insulin. Since both insulin and chronic stimulation by β-adrenergic agonists attenuate β-adrenergic action (29), we investigated if insulin treatment alters the level of β2AR sequestration from the cell membrane as does β-adrenergic agonist (2, 27, 28), measured using the hydrophilic, membrane-impermeant radioligand [HICGP-12177 (27). As shown previously (27), treatment of DDT, MF-2 hamster smooth muscle cells in culture with isoproterenol promotes an ~40% decline in the amount of receptor accessible by the hydrophilic [HICGP-12177 radioligand. Notably, treatment with insulin promoted a significant, but lesser internalization of the β2ARs (Fig. 1).

IGF-1, which also counter-regulates β2AR-mediated response (3–5), promotes internalization of the β2ARs (mean ± S.E., 35 ± 2%, n = 3). In the presence of either insulin or IGF-1, the internalization of β2AR in response to isoproterenol was increased. Stimulation by insulin and isoproterenol in combination promoted internalization of more than 55% of the β2ARs. These observations derived from studies with DDT, MF-2 smooth muscle cells were explored in CHO cells stably transfected to express wild-type β2ARs (Fig. 1). The data from multiple experiments from both the DDT, MF-2 cells and CHO β2AR-expressing cells were in good agreement, demonstrating that this growth factor-stimulated internalization of β2ARs is not unique to the smooth muscle cells.

The functional read-out of insulin action on cyclic AMP accumulation in response to stimulation by β-adrenergic agonist was measured in the DDT, MF-2 cells challenged with isoproterenol in the absence and presence of insulin (Fig. 2). The time course for isoproterenol-stimulated cyclic AMP accumulation was rapid, peaking at ~3 min. As noted earlier (3, 4), insulin (100 nM) counter-regulates the ability of isoproterenol to stimulate intracellular accumulation of cyclic AMP. Thus, insulin both counter-regulated β-adrenergic stimulation of cyclic AMP accumulation (Fig. 2) and stimulated internalization of the β2ARs (Fig. 1).

Upon tyrosine kinase receptor-catalyzed phosphorylation (3), the Tyr-350 residue located in the cytoplasmic, C-terminal tail of the β2AR creates an SH2 recognition domain capable of binding the adaptor protein Grb2 (4, 5). The Grb2 molecule displays two SH2 domains and one SH3 domain. The similarity of the M_c of Grb2 to that of the light chains of the IgG preloaded detection of Grb2 by immunoblotting of immunoprecipitates of

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Fig. 1. Insulin and IGF-1, as well as isoproterenol, promote internalization of $\beta_2$ARs. Cultures of DDT-MF-2 cells (left-hand panel) and of CHO clones stably transfected to express wild-type hamster $\beta_2$ARs (right-hand panel) were untreated or treated with insulin (100 nM) or IGF-1 (100 nM) in the presence or absence of isoproterenol (10 $\mu$M) or wortmannin (100 nM) for 5 min at 25 °C. The internalization of the receptor was measured using a hydrophilic, impermeant radiolabeled $\beta$-adrenergic antagonist $[^{3}H]$CGP-12177, as described (23). The data are mean values ± S.E. from either triplicates of a single experiment, representative of multiple experiments (left-hand panel) or five separate experiments (right-hand panel). Asterisks denote $p \leq 0.05$ for the difference with reference to the internalization in the unstimulated cells, arbitrarily set at 0%. $\beta_2$AR expression, as measured by $[^{125}$I]$\text{Iodocyanopindolol binding, for the CHO cells stably expressing} \beta_2$ARs is 114 ± 10 fmol/10^6 cells (mean value ± S.E., n = 4), comparable to the level of expression of the DDT-MF-2 hamster smooth muscle cells (28).

whole-cell extracts performed with antibodies to $\beta_2$ARs. As an alternative strategy to probing the possible direct association of Grb2 with $\beta_2$ARs to $\beta_2$ARs in the counter-regulatory actions of insulin, whole-cell extracts were subjected to immuneprecipitations with anti-Grb2 antibodies first or treated with insoluble beads to which a GST fusion protein (GST-Grb2) was immobilized. Immunoprecipitations performed with anti-Grb2 antibodies were subjected to SDS-PAGE and then immunoblotting with antibodies specific for $\beta_2$AR (Fig. 3). A time course of insulin action on Grb2 binding to $\beta_2$ARs was established. Shown in Fig. 3 are representative examples of blots from two time courses in which immunoprecipitates with anti-Grb2 antibodies were performed with extracts of cells challenged with insulin for 1–40 min, subjected to SDS-PAGE, and stained subsequently with antibodies to $\beta_2$ARs. Both trials reveal Grb2 binding to $\beta_2$ARs in the whole-cell extracts from untreated, control cells. Insulin treatment of the cells leads to an increase in the amount of $\beta_2$ARs associated with Grb2, peaking at ~5 min after challenge with insulin and declining thereafter. Association of $\beta_2$ARs with Grb2 was probed further using a complementary approach, i.e., challenging cells with either insulin or agonist (or both) and treating extracts prepared from these cells with a GST-Grb2 fusion protein immobilized to insoluble beads. Binding of $\beta_2$ARs to the Grb2 was detected in immunoblots of the proteins bound to the immobilized Grb2 stained with anti-$\beta_2$AR antibodies (Fig. 4); those data are in good agreement with the data obtained with immunoprecipitates performed with anti-Grb2 antibodies (Fig. 3). The amount of $\beta_2$AR associated with the GST-Grb2 fusion protein was quantified from several separate experiments and, as displayed in Fig. 4, was found to increase by more than 2-fold in extracts from those cells challenged with insulin. Isoproterenol, in contrast, stimulates only a small and variable increase in the amount of $\beta_2$AR-Grb2 binding. Challenge with isoproterenol and insulin, in combination, provokes an increase in the amount of $\beta_2$ARs associating with GST-Grb2 similar to that obtained with insulin alone. These data extend earlier studies on the role of tyrosine phosphorylation of $\beta_2$ARs in insulin action (3–5) and the ability of insulin to create a Grb2-dependent shift in agonist affinity of $\beta_2$ARs (28). The current studies provide a direct demonstration that the insulin-catalyzed phosphorylation of the $\beta_2$AR creates a bona fide SH2 domain that can be shown to bind Grb2 in an insulin-dependent manner, using two distinct but complementary approaches.

Dynamin, a GTPase that regulates the assembly and trafficking of clathrin-coated vesicles, has been shown to be an essential element in the sequestration of $\beta_2$ARs (23). The ability of either insulin alone or in combination with isoproterenol to increase Grb2 binding to $\beta_2$ARs provides through the Grb2 SH3 domain the opportunity to bind dynamin. The possible role of both dynamin and PI 3-kinase in the trafficking of membrane proteins and our observation that insulin induces internalization of $\beta_2$ARs prompted us to explore the extent to which insulin stimulates association of dynamin and Grb2, using adsporption to the GST-Grb2 fusion protein (Fig. 4). Insulin stimulates a 4.2 ± 0.7-fold (mean ± S.E., n = 3) increase in the amount of dynamin associated with Grb2. Isoproterenol stimulates a 2.7 ± 0.5-fold increase in the association of dynamin with Grb2, consistent with recent observations by Zhang et al. (23). In combination, insulin and isoproterenol stimulate a 6.3 ± 1.3-fold increase in dynamin association with GST-Grb2. Grb2 binding to the $\beta_2$AR operates through an SH2 domain on the $\beta_2$AR (29) created via phosphorylation of Tyr-350 in response to insulin (3–5), whereas the interaction of dynamin with Grb2 is presumed to be via SH3 domains.

Phosphatidylinositol 3-kinase plays a critical role in the traf-
flicking of membrane proteins. The ability of insulin to provoke internalization of β2ARs stimulated us to ascertain the association of PI 3-kinase with Grb2 in response to insulin and isoproterenol, both alone and in combination (Fig. 5). The p85 catalytic subunit of PI 3-kinase was readily detected in the GST-Grb2 adsorptions of extracts from control, untreated cells. Treatment with either insulin or isoproterenol yielded an increase in the amount of p85 binding to Grb2. Immunoprecipitations performed with antibodies to p85 subunit of PI 3-kinase associated with Grb2. The dose-response relationship for insulin-stimulated association of PI 3-kinase with Grb2 was established using direct measurement of PI 3-kinase activity. The PI 3-kinase activity displayed in immunoprecipitations performed with antibodies to β2ARs with whole-cell extracts of DDT-MF-2 smooth muscle cells challenged with insulin (0 to 100 nM) was determined (Fig. 5). The lowest concentration of insulin tested, 0.01 nM, was sufficient to stimulate a 2-fold increase in the amount of PI 3-kinase associated with the β2AR. Increasing the concentration of insulin to 1 nM increased the amount of PI 3-kinase activity displayed in immuneprecipitations performed on as many separate occasions.
3-kinase activity associated with the $\beta_2$ARs to 4.4-fold over basal. At the highest concentration of insulin tested (100 nM), PI 3-kinase activity associated with immunoprecipitates of $\beta_2$AR was increased ~7-fold over basal. By staining of immunoprecipitates of $\beta_2$ARs with anti-phosphotyrosine antibodies, earlier studies reported a dose-dependence relationship for insulin over this same range of concentrations (3–5).

The microbial product wortmannin is a potent inhibitor of PI 3-kinase activity (25). Inhibiting PI 3-kinase of DDT MF-2 smooth muscle cells by wortmannin revealed several new insights about the possible role of PI 3-kinase in growth factor–induced internalization of $\beta_2$ARs. Wortmannin abolished the insulin-stimulated increase in PI 3-kinase activity associated with the $\beta_2$AR (Fig. 5) and blocked the ability of insulin to counter-regulate $\beta$-adrenergic stimulation of cyclic AMP accumulation (Fig. 2). The PI 3-kinase inhibitor had no effect on the internalization of $\beta_2$AR in response to the $\beta$-adrenergic agonist isoproterenol (data not shown). In contrast, wortmannin reduced by ~50% the amount of $\beta_2$ARs internalized in response to stimulation by isoproterenol and insulin in combination as compared with that internalized by isoproterenol alone, 63 ± 4% internalization of $\beta_2$ARs in response to insulin+isoproterenol together as compared with 34 ± 2% internalization of $\beta_2$ARs in response to isoproterenol alone (mean value±S.E., $n = 3$). IGF-I (100 nM) in combination with isoproterenol provoked internalization of 50 ± 3% ($n = 3$) of $\beta_2$ARs (Fig. 2), and this internalization of $\beta_2$ARs was sensitive to inhibition by wortmannin. Thus, wortmannin blocked insulin-stimulated PI 3-kinase activity, the ability of insulin to counter-regulate $\beta$-adrenergic stimulation of cyclic AMP accumulation, and that component of $\beta_2$AR internalized in response to insulin when compared with those stimulated by both isoproterenol and insulin together.

To provide an additional test of the hypothesis linking insulin-stimulated phosphorylation of tyrosyl residue 350 to the $\beta_2$AR with Grb2 and internalization of $\beta_2$ARs, we made use of CHO clones stably expressing the $\beta_2$AR. As shown in Fig. 6, when performed with immobilized GST-Grb2 and extracts from CHO clones expressing wild-type $\beta_2$AR, immunoblotting reveals a time-dependent association of $\beta_2$AR with Grb2 in response to insulin (100 nM). These data agree well with those studies performed by immunoprecipitations with anti-Grb2 antibodies then subjected to SDS-PAGE and staining with anti-$\beta_2$AR antibodies (Fig. 3) and with the earlier studies employing this same approach (Fig. 4). Parallel experiments performed with CHO clones expressing the Y350F mutant form of the $\beta_2$AR. As shown in Fig. 6, when performed with immobilized GST-Grb2 and extracts from CHO clones expressing wild-type $\beta_2$AR, immunoblotting reveals a time-dependent association of $\beta_2$AR with Grb2 in response to insulin (100 nM). These data agree well with those studies performed by immunoprecipitations with anti-Grb2 antibodies then subjected to SDS-PAGE and staining with anti-$\beta_2$AR antibodies (Fig. 3) and with the earlier studies employing this same approach (Fig. 4). Parallel experiments performed with CHO clones expressing the Y350F mutant form of the $\beta_2$AR revealed no detectable association of the Y350F $\beta_2$ARs with the GST-Grb2 (Fig. 6). The ability of the Y350F $\beta_2$ARs to be internalized in response to insulin was tested. The Y350F mutation abolished internalization in response to insulin. Insulin-stimulated internalization of $\beta_2$ARs was 31 ± 3.3% for CHO clones stably expressing wild-type $\beta_2$AR, whereas the amount of $\beta_2$AR internalized in response to insulin was only 2 ± 3.5% for the CHO clones expressing the Y350F mutant form of $\beta_2$AR (mean value±S.E., $p = 0.01$ for the difference, $n = 4$). Earlier, it was shown that the Y350F mutation of the $\beta_2$AR attenuates the ability of insulin to counter-regulate catecholamine-stimulated cyclic AMP accumulation (4). These studies demonstrate the critical role of insulin-stimulated phosphorylation of the Tyr-350 residue of the $\beta_2$AR for...
Receptor phosphorylation and sequestration are prominent features of agonist-induced desensitization of GPLRs (for review, see Refs. 2, 29, and 30). TKRs, e.g., those for insulin or IGF-1, counter-regulate actions of one prominent member of the GPLRs, the β2AR (3), via phosphorylation (4, 5), but the possible involvement of β2AR sequestration in their action was untested. Herein we have demonstrated several important features about the counter-regulatory effects of insulin on the β2AR. Insulin (and IGF-1) stimulate internalization of β2ARs, as tested in two independent cell lines. TKRs have been shown to catalyze phosphorylation of the β2AR (3–5). Insulin stimulates in a rapid, dose-dependent manner Grb2 binding, a prerequisite for association of the β2AR-Grb2 complex with proteins that bind to the SH3 domain of Grb2, such as dynamin and PI 3-kinase. Mutation of Y350F abolishes the following: 1) the ability of β2AR to associate with Grb2, 2) the insulin-stimulated sequestration of β2ARs, and 3) the counter-regulatory effects of insulin on β-adrenergic stimulation of cyclic AMP accumulation. Stimulation of cells by insulin (or IGF-1) and the β-adrenergic agonist isoproterenol promotes sequestration of β2ARs that is additive, and this component is sensitive to inhibition by the PI 3-kinase inhibitor wortmannin. β-Agonist-induced sequestration alone, in contrast, is largely unaffected by wortmannin, suggesting that it is not dependent upon PI 3-kinase activity. Elevation of intracellular cyclic AMP (31) or activation of protein kinase C (32) inhibits PI 3-kinase activity. Since β-adrenergic agonists stimulate accumulation of cyclic AMP as well as internalization of β2ARs in DDT1-MF2 cells, PI 3-kinase would not appear to mediate β-adrenergic agonist-induced internalization of β2ARs, in agreement with failure of wortmannin to influence this same process (Fig. 7). Likewise, stimulation by the catecholamine epinephrine (a mixed α- and β-adrenergic agonist) activates both protein kinase C, protein kinase A, and β-adrenergic receptor kinase in a variety of cells that demonstrate agonist-induced internalization of β2ARs (2). The ability of insulin in combination with β-adrenergic agonist to promote an internalization of β2ARs that is blocked by wortmannin argues for the existence of a wortmannin-sensitive PI 3-kinase activity that is insensitive to elevation of intracellular cyclic AMP alone (31).

Sequestration of β2ARs appears to involve a clathrin-mediated (27) and rab5-regulated (33) endocytic pathway (Fig. 7). PI 3-kinase has been shown to be involved in rab5-mediated early endosome fusion, which is sensitive to inhibition of PI 3-kinase by wortmannin (34). PI 3-kinase has been implicated also in G-protein-mediated activation of mitogen-activated protein kinase (35). Much like IRS-1 associates with PI 3-kinase in trafficking of the hexose transporter GLUT4 in response to insulin (18), the β2AR phosphorylated in response to TKRs may associate with PI 3-kinase during its own insulin-stimulated sequestration (Fig. 7). PI 3-kinase may be required for the TRK-stimulated sequestration process, whereas the targeted molecule, e.g., β2AR or the insulin-regulated hexose transporter GLUT4, may itself possess determinants obligate for trafficking to a sequestered site.

The counter-regulatory effects of insulin require phosphorylation of Tyr-350 of the β2AR (3, 4) and association of Grb2 (and thereby PI 3-kinase), whereas β-adrenergic agonist-induced desensitization by isoproterenol appears to operate independently of a wortmannin-sensitive PI 3-kinase (39). Insulin and, to a lesser extent, serum induce a Grb2-dependent shift in the agonist affinity of β2ARs (28). The insulin-stimulated Grb2-mediated effect on agonist affinity of the β2ARs requires treatment of the cells with insulin and is lost by the Tyr-to-Phe mutation of the Tyr-350 residue, by disruption of the SH2 but not SH3 domains of Grb2, and by introduction of the phosphoTVNpeptide, which blocks binding to SH2 domains (28). Phosphorylation of Tyr-350 residue of the β2AR in response to insulin (3, 4) and of other sites of the β2AR by protein kinase A, protein kinase C, or GRKs may operate as a coincidence detector for the combined actions of insulin and β-adrenergic agonists, both promoting receptor sequestration, but via two distinct yet interacting pathways (Fig. 7). The counter-regulatory effects of insulin on catecholamine action at the level of β2-adrenergic receptor sequestration were explored in the CHO cells and the DDT1-MF2 hamster smooth muscle cells in the current work. Major sites of insulin action, such as adipose tissue, liver, and skeletal muscle are prominent loci for counter-regulatory effects of insulin on β-catecholamine action, and we suspect insulin-stimulated sequestration of β2-adrenergic receptors to contribute to this physiological regulation at these sites. Efforts in progress to express the Y350F mutant β2-adrenergic receptor in these target tissues of transgenic mice will provide a direct test of the hypothesis.

Earlier work by others suggested that Tyr-141 was a major site for phosphorylation of the human β2AR in CHO cells stably expressing the human β2ARs (36). These cells were challenged in these earlier studies, however, with high concentrations of...
insulin (36). Using both metabolic labeling as well as matrix-assisted laser desorption ionization mass spectrometry, the Tyr-141 of the β2AR has been established as the major site of IGF-1-and not insulin-stimulated phosphorylation (37). The high concentration of insulin used earlier by these researchers, however, favors activation of IGF-1 receptors, and the high abundance of the IGF-1 receptors in these cells (35) provides a likely explanation for this apparent discrepancy (37). Clearly, Tyr-350 of the β2AR is a major site of insulin action and is shown to be critical to the insulin-stimulated binding of Grb2 by the β2AR and to TKR-stimulated internalization of the β2AR. Sequestration of β2ARs is shown to be a novel mechanism by which a growth factor, such as insulin or IGF-1, can counter-regulate the actions transduced via a G-protein-linked receptor, such as the β2AR.

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