Insulin activation of mitogen-activated protein kinases Erk1,2 is amplified via beta-adrenergic receptor expression and requires the integrity of the Tyr350 of the receptor.

Hsien-yu Wang1, Sergey Doronin2 and Craig C. Malbon2

1Department of Physiology & Biophysics, Diabetes & Metabolic Diseases Research Program, University Medical Center, SUNY/Stony Brook, Stony Brook, NY 11794-8661 and the 2Department of Molecular Pharmacology, University Medical Center, SUNY/Stony Brook, Stony Brook, NY 11794-8651

Key words: Erk1,2; beta-adrenergic receptor; insulin action; mitogen-activated protein kinase; phosphotyrosine, Tyr 350.

Running title: Insulin activation of ERK1,2 via β2-adrenergic receptors

4Corresponding author: C. C. Malbon, Pharmacology-HSC, SUNY/Stony Brook, Stony Brook, NY 11794-8651; voice phone 516-444-7873; FAX 516-444-7696; e-mail craig@pharm.som.sunysb.edu
Summary

Insulin activates a complex set of intracellular responses, including the activation of mitogen-activated protein kinases Erk1,2. The counterregulatory actions of insulin on catecholamine action are well-known and include phosphorylation of the β2-adrenergic receptor on Tyr 350, Tyr354 and Tyr364 in the C-terminal, cytoplasmic domain as well as enhanced sequestration of the β2-adrenergic receptor. Both β-adrenergic agonists and insulin provoke sequestration of β2-adrenergic receptors, in a synergistic manner. In the current work, crosstalk between insulin action and β2-adrenergic receptors revealed that insulin activation of Erk1,2 was amplified via β2-adrenergic receptors. In Chinese hamster ovary cells, expression of β2-adrenergic receptors enhanced 5-10-fold the activation of Erk1,2 by insulin and prolonged the activation, the greatest enhancement occurring at 5 min post insulin. The potentiation of insulin signaling on Erk1,2 was proportional to the level of expression of β2-adrenergic receptor. The potentiation of insulin signaling requires the integrity of Tyr350 of the β2-adrenergic receptor, a residue phosphorylated in response to insulin. β2-adrenergic receptors with a Tyr350Phe mutation failed to potentiate insulin activation of Erk1,2. Expression of the C-terminal domain of the β2-adrenergic receptor (Pro323-Leu418) in cells expressing the intact β2-adrenergic receptor acts as a dominant negative, blocking the potentiation of insulin activation of Erk1,2 via the β2-adrenergic receptor. Blockade of β2-adrenergic receptor sequestration does not alter the ability of the β2-adrenergic receptor to potentiate insulin action on Erk1,2. We propose a new paradigm in which a G-protein-linked receptor, such as the β2-adrenergic receptor, itself acts as a receptor-based scaffold via its binding site for SH2 domains, facilitating signaling of the mitogen-activated protein kinase pathway by insulin.
Introduction

G-protein-linked receptors (GPLR) and growth factor receptors with intrinsic tyrosine kinase activity (TKR) represent two prominent pathways for cellular signaling (1;2). Study of the integration of signaling between GPLR and TKR pathways has revealed recently the existence of crosstalk at the most proximal point, receptor-to-receptor interaction with GPLRs acting as substrates for TKRs (3-5). Insulin stimulates the phosphorylation of the β2-adrenergic receptor (β2AR) on tyrosyl residues Y350/354 and Y364, both in vivo (3;4) and in vitro (5) using recombinant, purified β2AR and insulin receptors. Tyrosyl residue 350, a prominent residue for insulin receptor-catalyzed phosphorylation, is embedded in a sequence motif (Tyr-Gly-Asn-Gly) which is similar to the motifs known to interact with C. elegans sem5 Src homology 2 (SH2) domains when phosphorylated (6). Phosphorylation of sites on the β2AR by the insulin receptor and the IGF-1 receptor include a motif for TKR at Y364 (7), the Grb2 binding site at Y350 (4;8), and a potential SHC binding site at Y132 (4;5;7). For insulin action, activation of 1-phosphatidylinositol 3-kinase (PI3K) is an early event, following temporally the phosphorylation of the insulin receptor and IRS-1 (9;10). In response to insulin stimulation, the p85 regulatory subunit of PI 3-kinase binds the IRS-1 via SH2 domain(s), activating the catalytic p110 subunit which phosphorylates various phosphoinositides at the 3'-position of the inositol ring (11). Ample reports support the premise that PI 3-kinase and its 3'-phosphoinositide products are critical to intracellular trafficking of membrane-bound elements in general (12) and of downstream elements of TKR signaling, particularly insulin (13). We have shown that insulin, much like β-adrenergic agonists, provoke rapid sequestration of β2AR, in a synergistic manner (14;15). We probed for possible cross-talk between insulin and β2ARs in the mitogen-activated protein kinase pathway. Remarkably, the activation of Erk1,2 by insulin in CHO cells was found to be amplified by β2AR expression, i.e., the higher the level of cellular complement of β2AR, the greater was the potentiation of insulin activation of Erk1,2. Further studies reveal that the ability of the β2AR to amplify the insulin response was dependent on the integrity of the Tyr350 by guest on March 22, 2020 http://www.jbc.org/ Downloaded from
residue, which is phosphorylated by the insulin receptor and constitutes a binding site for an SH2 domain to which Grb2, and other proteins, can bind (16). A new paradigm is proposed in which G-protein-linked receptors function as a receptor-based scaffold via a binding site for SH2 domains, amplifying signaling via the mitogen-activated protein kinase pathway.

**Experimental Procedures**

*Materials*—Chinese hamster ovary (CHO) stably expressing wild-type and mutant β2ARs and human epidermoid carcinoma A431 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% and 10% fetal bovine serum, respectively (17-20). Prior to analysis for insulin action, the cells were serum-starved overnight (4). The CHO clones stably expressing the hamster β2AR were characterized earlier (17). The C-terminal, cytoplasmic domain of the hamster β2AR (Pro323-Leu418) was cloned into the pCDNA3 expression vector and employed to stably transfect A431 clones.

*Assay of activation of Erk1,2*—Cells were stimulated with EGF (50 ng/ml) or insulin (100 nM) for the indicated times and then lysed (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH2PO4, 10 mM Na-molybdate, 2 mM Na-orthovanadate, 20 mM Tris-HCl [pH 7.4], 1% Triton X-100, 0.5% NP-40, 6 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml Leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride). Samples were subjected to SDS-PAGE and immunoblotting as described earlier (21). The blots were stained with antibodies purchased from Promega (Madison, WI) that specifically recognize only the dually phosphorylated, active forms of Erk1,2. For inhibitor studies, A431 cells and CHO clones stably transfected to express β2-adrenergic receptors were pretreated for 12 hours in the absence or presence of one of the following agents: the PI3K inhibitor LY294002 (LY, 20 μM), the MEK inhibitor PD98059 (PD, 10 μM), or the Src inhibitor PP2 (PP2, 50 nM). After an overnight exposure to an inhibitor, the cells were incubated with or without 100 nM insulin for 5 min and the amount of Erk1,2 activated measured.
**Radioligand Binding Studies** – The number of β2AR was determined by radioligand binding. Intact A431 cells were incubated with 0.5 nM [125I]iodocyanopindolol (ICYP; NEN Life Science Products, Boston, MA) in the presence or absence of 10 µM propranolol at 23°C for 90 min. The incubation buffer contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 150 mM NaCl. The cells were collected on GF/C membranes at reduced pressure and washed rapidly. The radioligand bound to the washed cell mass retained by the filter was quantified by use of a γ-counter (17).

**Sequestration of β₂-adrenergic Receptor** – Receptor sequestration was assayed using the hydrophilic, membrane-impermeable β-adrenergic antagonist [3H]CGP-12177(18). A431 cells were preincubated with isoproterenol (10 µM) for periods up to 60 min, or preincubated with insulin (100 nM) for 5 to 30 min. The cells then were resuspended in DMEM containing 20 mM HEPES, pH 7.4, and 70 nM [3H]CGP-12177 (NEN Life Science Products, Boston, MA) at 4°C for 6 h. The cells were diluted, collected on GF/C membranes, and washed rapidly. The radioligand bound to the washed cell mass retained on the filter was counted by use of liquid scintillation spectrometry. Nonspecific binding was defined as the radioligand binding insensitive to competition by the unlabeled, β-adrenergic antagonist propanolol (10 µM).

**Confocal Microscopy** – A431 cells stably transfected with green fluorescence protein (GFP)-tagged β₂-adrenergic receptor were treated with or without insulin, in the absence or presence (overnight) of the PI3K inhibitor LY294002 (20 µM). The cells were incubated with or without 100 nM insulin for 5 min, fixed with 3% paraformaldehyde, and washed 3 times with MSM-Pipes buffer (18 mM MgSO₄, 5 mM CaCl₂, 40mM KCl, 24 mM NaCl, and 5 mM Pipes, pH 6.8). The cells were analyzed by confocal laser scanning microscopy on an Odyssey instrument (Noran Instruments). The construct pCDNA3-β₂AR-GFP, encoding a β₂-adrenergic receptor fusion protein with GFP at its carboxyl terminus (22) was a generous gift from Dr. J. L.
Benovic. The confocal microscopy was performed at the University Microscopy Imaging Center at Stony Brook.

Data Presentation and Analysis – Unless otherwise noted, the values presented are means ± S.E.M. The autoradiograms are representative of multiple (at least three), independent experiments. In all figures, an “**” denotes a mean value with statistical significance ($p \leq 0.05$) compared to the mean values of the control, time-zero or to the control group, as indicated in the legend to the figure.

Results

Initial studies of the time-course for activation of Erk1,2 revealed a biphasic response to insulin stimulation of CHO clones. In CHO-K cells, which express very low numbers of endogenous β2ARs, the activation of Erk1,2 displayed an initial peak of activation (as measured with phospho-specific antibodies for activated Erk1,2) in response to insulin at 0.5-1.0 min (Fig. 1 inset), followed by a gradual decline in activation observed in analyses of multiple experiments performed on separate occasions (Fig. 1, bar graph). The activation of Erk2 (p42Erk) in response to insulin exceeded that of Erk1 (p44Erk). When examined in CHO cells expressing a modest level of β2ARs (18.2 fmol/100,000 cells), the activation of Erk1,2 in response to insulin shows both a biphasic response as well as an amplified response to stimulation by insulin. The Erk1,2 response to insulin showed an early (0.5-1.0 min) peak of activation followed by a robust later response, peaking at 5 min, declining to unstimulated levels within 10 min (not shown). The response for activation of p42Erk in the CHO-β2AR clones was 5-7 fold greater than that in the β2AR-deficient CHOK clones. The response for activation of p44Erk by insulin was similar to that observed for p42Erk, i.e., 5-7 fold greater in the CHO-β2AR clones as compared to the CHOK clones. The simplest interpretation of the data is that the expression of β2AR potentiates and prolongs the ability of insulin to activate Erk1,2, a novel hypothesis.
To test the hypothesis that the expressed level of the G-protein-linked receptor β2AR regulates the temporal nature and magnitude of the Erk1,2 activation by insulin, we examined the Erk1,2 response to activation by insulin in cells that stably express varying levels of β2AR (Fig. 2). Four clones were included in the analysis that displayed few receptors (CHOK, wild-type; WT, 0.6 ± 0.08 fmol/100,000 cells), a low (βL, 4.5 ± 1.1 fmol/100,000 cells), middle (βM, 18.3 ± 2.3 fmol/100,000 cells), and high (βH, 27.9 ± 3.0 fmol/100,000 cells) level of expressed β2AR (ICYP binding, mean values ± SEM, n=3). The activation of Erk1,2 was measured in response to insulin challenge after 5 min. Immunoblots of the cell lysates stained with antibodies to the phospho-specific, activated forms of Erk1 and Erk 2. In the absence of insulin, levels of activated Erk1,2 were very low. Often, as shown here, the basal levels of phosphorylated, activated Erk1,2 were suppressed in the clones expressing β2AR. The greater the expression of β2AR, the greater was the apparent suppression of basal levels of Erk1,2 activation. Challenge with epidermal growth factor (EGF) provokes an activation of Erk1,2 in WT cells with low levels of β2AR, whereas the EGF response is attenuated in the clones expressing significant levels of β2ARs. The Erk1,2 activation in response to insulin, in sharp contrast, is clearly amplified in cells expressing β2ARs. Although not strictly proportional, the activation of Erk1,2 by insulin, but not EGF, was amplified to a greater extent with increasing levels of β2AR expression. These data suggest that the β2AR appears to facilitate insulin signaling through the mitogen-activated protein kinase cascade to the level of Erk1,2.

It was important to evaluate whether or not co-stimulation of the insulin- and β2AR-stimulated pathways leads to activation of Erk1,2 and whether or not some adrenergic agonist activity derived from serum might contribute to the insulin response. Treating A431human epidermoid carcinoma cells with isoproterenol (10 µM) or insulin (100 nM) stimulated the activation of both Erk1 and Erk2 (Fig. 3). Insulin and isoproterenol, in combination, do not produce an additive response. Propranolol (10 µM) blocked isoproterenol-stimulated activation
of Erk1,2, but not the response to stimulation by insulin. These data rule out the possibility that co-stimulation by some serum-derived agent via a β2AR-mediated pathway is an explanation for the enhanced insulin-stimulated activation of Erk1,2 in cells expressing β2AR. The diterpene activator of adenylyl cyclase forskolin stimulates cyclic AMP accumulation and also provoked a weak activation of p44Erk and an activation of p42Erk similar to that of isoproterenol. These same results were obtained in CHO clones stably transfected to express β2ARs (data not shown). The inverse agonist compound ICI118551 was without effect on Erk1,2 activation in these cells (data not shown).

We extended these studies by examining the ability of insulin to activate Erk1,2 in A431 cells that were challenged simultaneously with either 1 or 10 μM isoproterenol (Fig. 4). The response to insulin alone is provided for comparison. Treatment with 1 μM isoproterenol alone provokes activation of Erk1,2. Increasing the concentration of insulin leads to further activation of Erk1,2. Increasing the concentration of isoproterenol alone to 10 μM leads to additional activation of that produced by 1 μM. Challenging the A431 cells with 10 μM isoproterenol in combination with increasing concentrations of insulin leads to a dampening of the activation of Erk1,2, especially at the lower concentrations of insulin (1 and 10 nM). These data suggest that the ability of isoproterenol to activate Erk1,2 is intrinsically lower than that of insulin to activate Erk1,2. In combination, insulin and isoproterenol do not activate Erk1,2 in an additive manner, but rather appear to compete for the activation process in a weakly competitive manner.

The observations both in vivo (4;23) and in vitro (5) demonstrate that insulin treatment leads to the phosphorylation of the β2AR on tyrosyl residues, particularly Tyr350. Phosphorylation of Tyr350 creates a binding site for SH2 domains that can act as a protein module for the docking and regulation of a wide-spectrum of adaptor molecules as well as interesting molecules such as Src and PI3 kinase (6). The ability of insulin to counterregulate the ability of the β2AR to activate adenylylcyclase is dependent upon the availability of the
Tyr350 for phosphorylation. Mutation of this tyrosyl residue abolishes the counterregulation of β2AR-stimulated cyclic AMP accumulation (4;24). Similarly, the ability of insulin to provoke profound sequestration of the β2AR is dependent upon the availability of Tyr350, i.e., the Tyr350Phe mutant fails to display insulin-induced sequestration (14). We tested if mutation of the wild-type β2AR to Tyr350Phe, likewise, might influence the ability of insulin to signal to the level of Erk1,2 activation (Fig. 5). As displayed in the immunoblot of whole-cell extracts stained with antibodies to the phospho-specific, activated forms of Erk1,2, insulin stimulates an enhanced activation of Erk1,2 in CHO cells expressing the wild-type (Y350) β2AR. In order to address the nature of the effect of expressing a mutant receptor on the insulin response, we selected clones which express nearly equivalent levels of β2AR (3.5 ± 0.7 and 3.0 ± 0.6 fmol/100,000 cells for mutant Tyr350Phe and wild-type β2AR-expressing cells, respectively). The effect of the Tyr350Phe mutation was clear, the activation of Erk1,2 by insulin in the cells expressing the mutant receptors was largely abolished. Analysis of data from multiple experiments confirms the data displayed in the immunoblot. Mutation of Y350 of β2AR blocks the ability of insulin to counterregulate β2AR-mediated activation of adenylyl cyclase (3;4) and to stimulate sequestration of the β2AR (14), as well as the ability of the insulin receptor to phosphorylate Y350 both in vivo (4) and in vitro (5). Taken together, these results further implicate the β2AR acting as a receptor-based scaffold for insulin signaling in which phosphorylation of the Tyr350 residue of the β2AR creates a binding site for SH2 domains in response to insulin, a key element to activation of Erk1,2.

The Try350 residue is located in the C-terminal, cytoplasmic tail of the β2AR. If the β2AR is acting as a template or scaffold for insulin signaling to the level of Erk1,2, then expression of the C-terminal domain of the receptor which is not localized to the plasma membrane may influence this novel ability of the β2AR to potentiate insulin signaling. Human epidermoid A431 cells were employed due to their relatively high native expression of β2AR.
A431 clones were stably transfected with an expression vector (pCDNA3) harboring the entire, cytoplasmic C-terminal domain of the β2AR (BAC-1). Expression was confirmed for the A431 cells stably transfected to express BAC1 by accumulation of the peptide (Mr=11 kDa) in sufficient quantities to permit ready detection in immunoblots of the whole-cell extracts from these cells, probed with an antibody to BAC1 (Fig. 6A). Insulin challenge of A431 cells yields a significant activation of Erk1,2 (Fig. 6B). The expression of BAC-1 in A431 clones resulted in a total loss of the ability of insulin to signal to the level of Erk1,2. Probing immunoblots of extracts from the A431 clones with antibodies specific for phosphotyrosine (Anti-pY) revealed, indeed, that BAC1 was tyrosine phosphorylated in response to insulin (Fig. 5A). Expression of BAC1 had no effect on the ability of isoproterenol (10 µM) to stimulate a normal elevation of intracellular cyclic AMP accumulation by A431 cells (data not shown). Several slower migrating species that are recognized by the BAC1 antibody and are phosphorylated in response to insulin are likely to represent palmitoylated forms of BAC1(25). Thus, with respect to the activation of Erk1,2 by insulin in cells expressing native β2AR, BAC-1 acts as a dominant-negative molecule that is phosphorylated in response to insulin stimulation and suppresses the ability of insulin to signal.

PI3K plays an important role in many cellular processes, including intracellular trafficking of molecules (26;27). The microbial product wortmannin and the LY294002 compound both inhibit PI3K and many facets of intracellular trafficking. Inhibitors of P13K block various aspects of insulin action and we evaluated if the LY294002 compound would influence the ability of the β2AR to enhance insulin activation of Erk1,2 and perhaps influence the sequestration of β2ARs (Fig. 7). Insulin, as well as isoproterenol, stimulates the sequestration of β2AR(14). The cellular localization of the β2AR was defined by use of a green fluorescent protein (GFP)-tagged version of the β2AR, characterized earlier (18;22). Using A431 cells stably transfected with an expression vector harboring the GFP-tagged β2AR in tandem with confocal laser-scanning microscopy, we observed the GFP-tagged β2AR display a pattern of distribution largely
confined to the cell membrane (Fig. 7, panel a). Challenge with insulin induces a dramatic sequestration of the β2AR from the cell membrane to intracellular locales (Fig. 7, panel b), as previously observed (14). In the presence of the LY294002 inhibitor (20 µM), in contrast, the ability of insulin to induce sequestration of the β2AR was abolished (Fig. 7, panel c). Analysis of β2AR internalization using the hydrophilic, beta-adrenergic antagonist ligand [3H]CGP-12177, confirms in an independent manner that inhibition of PI3K with the LY compound suppresses insulin-stimulated β2AR sequestration, measured at 5 min post insulin (Table 1). Taken together, these data suggest that the ability of insulin, but not the ability of isoproterenol, to stimulate sequestration of the β2AR was dependent upon PI3K, i.e., inhibition of PI3K by LY compound suppresses sequestration of β2AR in response to insulin. These novel data prompted us to probe further the possible role for β2AR internalization in the ability of β2AR to potentiate insulin activation of Erk1,2.

To probe further the potentiation of insulin signaling to Erk1,2 by β2AR, we tested inhibitors of Src, MEK, and PI3 kinase. The studies were performed in both A431 cells and CHO clones stably transfected to express β2AR. Recent data have implicated the non-receptor tyrosine kinase Src in the biology of β2AR (28), for this reason the PP2 inhibitor of Src was tested first. Treatment with the PP2 inhibitor (50 nM) failed to influence the ability of cells expressing β2AR to potentiate insulin activation of Erk1,2 (Fig. 8). The MEK inhibitor PD98059 (29) (10 µM, overnight), in sharp contrast, abolishes all activation of Erk1,2, suggesting that the potentiation of insulin-stimulated Erk1,2 activation by β2AR is, in fact, mediated via MEK.

The LY294002 compound, which inhibits 1-phosphatidylinositol 3-kinase, was without effect on the ability of β2AR to potentiate Erk1,2 activation by insulin (Fig. 8), although LY294002 was found to suppress insulin-stimulated β2AR sequestration (Fig. 7). We wondered if inhibition of PI3K activity and blockade of β2AR sequestration might alter either the time-course or decay of the activation of Erk1,2 by insulin. A431 cells pretreated with the LY
compound for 2 hours prior to challenge with insulin display the same onset, activation, and decay of the response as cells not treated with LY294002 (Fig. 9).

The results suggest that insulin and isoproterenol can both activate Erk1,2 independently of each other, but that there activation in combination is non-additive. To test further the notion that these two agents may compete on one level with each other for the activation of Erk1,2, we investigated the effects of pretreating A431 cells with isoproterenol for 30 min on the ability of a subsequent challenge of insulin to activate Erk1,2. Cells were challenged with isoproterenol for 30 min and then challenged directly without or with increasing concentrations of insulin (Fig. 10). When challenged sequentially, the ability of isoproterenol to counterregulate the activation of Erk1,2 by insulin was revealed. The activation of Erk1,2 by insulin was diminished in cells challenged 30 min prior with isoproterenol. When challenged simultaneously, this ability of isoproterenol to counterregulate insulin action was not so obvious (Figs. 3 and 4).

Discussion

The counterregulatory effects of catecholamines on insulin action are well known. We reveal the activation of Erk1,2 to be the target of both insulin and beta-adrenergic agonists, with beta-adrenergic agonists and insulin, at one level, competing for β2AR (Fig. 11). Since the extent of Erk1,2 activation is less for beta-adrenergic agonists than for insulin, this competition dictates the final level of Erk1,2 activity, which in turn can modulate other members of the mitogen-activated protein kinase network. On another level, the current work identifies a novel role for the β2AR and perhaps other GPLRs, i.e., acting as a receptor-based scaffold enhancing the signaling of other pathways. Expression of the β2AR clearly can potentiate the ability of insulin to activate Erk1,2. The evidence to support this notion is as follows: activation of Erk1,2 by insulin is potentiated several fold and prolonged in cells expressing elevated levels of β2AR; the extent of the potentiation of insulin action on Erk1,2 correlates with the amount of β2AR
expressed; mutation of the β2AR tyrosyl residue (Tyr350Phe) that is both phosphorylated in response to insulin and creates a binding site for SH2 domains abolishes the potentiation of insulin action; the C-terminal, cytoplasmic domain of the β2AR (BAC1), when expressed in cells is phosphorylated in response to insulin and acts as a dominant-negative with respect to enhanced activation of Erk1,2 by insulin; and, blockade of β2AR internalization does not suppress the ability of the β2AR to potentiate insulin activation of Erk1,2. Several well-known GPLRs themselves activate the Erk1,2 pathway(2). In some cases, the activation of Erk1,2 requires internalization of the GPLR, whereas in others the activation of Erk1,2 proceeds in the absence of internalization (30;31). The ability of the β2AR to crosstalk to and potentiate insulin action on Erk1,2 also does not require large-scale β2AR sequestration.

GPLR-based scaffold functions have been implicated for direct effects of GPLR-agonists action on the mitogen-activated protein kinase network (28). We propose that the potentiation of insulin activation of Erk1,2 is yet another example of a GPLR-based scaffold. The β2AR, upon phosphorylation in response to insulin (3-5;23;24), possess a binding site for SH2 domains (5) that can interact with a variety of adaptor molecules and enzymes, including PI3K and dynamin (Dyn) that are involved in β2AR sequestration (16). The sequestration of β2AR in response to agonist requires GRK-catalyzed phosphorylation, arrestin binding, and the involvement of clathrin, dynamin, and Src. The activation of Erk1,2 by insulin as compared to isoproterenol shares many common features (Fig. 11), and some interesting differences. Both insulin as well as beta-adrenergic agonists stimulate phosphorylation of the β2AR, the former by intrinsic tyrosine kinase activity of the insulin receptor (IRTK) and the latter by G-protein-linked receptor kinases (GRK). Both beta-adrenergic agonists and insulin can sequester β2AR into a vesicle-associated form and activate Erk1,2 independently. The sequestration by insulin, but not that by isoproterenol, is sensitive to the PI3K inhibitor LY294002 (LY). Blockade of insulin-induced sequestration by LY294002, however, does not block activation of Erk1,2 by insulin. The extent
of the activation of Erk1,2 is greater for stimulation by insulin than it is for beta-adrenergic agonists. Pretreatment with isoproterenol for 30 min leads to a diminished capacity of insulin to activate Erk1,2, suggesting that at one level the two pathways compete for $\beta_2$AR.

Taking these observations into account, we speculate that the $\beta_2$AR acts as a receptor-based scaffold for the MEKK, MEK, and/or Erk1,2 elements of the pathway, targeting one or more of these elements to the cell membrane. Although insulin clearly sequesters $\beta_2$AR, the activation of Erk1,2 proceeds even when the internalization is blocked, suggesting that the tyrosine-phosphorylated $\beta_2$AR may organize the elements leading to Erk1,2 activation. Scaffold proteins, such as the AKAP 250 gravin, have been shown only recently to play an integral role in the signaling of GPLRs (16;18-20). Expression of the $\beta_2$AR potentiates and prolongs the insulin signaling event and mutation of the Tyr350 residue abolishes the binding site for SH2 domains as well as the potentiation. Insulin stimulates the tyrosine phosphorylation and inactivation of the $\beta_2$AR with respect to activation of adenylylcyclase (and cyclic AMP accumulation), while simultaneously providing a scaffold via a binding site for SH2 domains on the $\beta_2$AR that can facilitate a second wave of signaling to the activation of Erk1,2. This proposal complements the notion that GPLRs can act as scaffolds for transactivation of receptor tyrosine kinases (2;28), only in this case the tyrosine kinase creates a GPLR-based scaffold via protein phosphorylation.

References

1. Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203-212
2. Morris, A.J. and Malbon, C.C. (1999) Physiological Reviews 79, 1373-1430
3. Hadcock, J.R., Port, J.D., Gelman, M.S., and Malbon, C.C. (1992) Journal of Biological Chemistry 267, 26017-26022
4. Karoor, V., Baltensperger, K., Paul, H., Czech, M.P., and Malbon, C.C. (1995) Journal of Biological Chemistry 270, 25305-25308
5. Baltensperger, K., Karoor, V., Paul, H., Ruoho, A., Czech, M.P., and Malbon, C.C. (1996) *Journal of Biological Chemistry* **271**, 1061-1064

6. Pawson, T. and Scott, J.D. (1997) *Science* **278**, 2075-2080

7. Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., and Lechleider, R.J. (1993) *Cell* **72**, 767-778

8. Geahlen, R.L. and Harrison, M.L. (1989) *Peptides and Protein Phosphorylation* 239-254

9. White, M.F. and Kahn, C.R. (1994) *Journal of Biological Chemistry* **269**, 1-4

10. Quon, M., Butte, A., and Taylor, S. (1994) *Trends in Endocrinology and Metabolism* **5**, 369-376

11. Rordorf-Nikolic, T., Van Horn, D.J., Chen, D., White, M.F., and Backer, J.M. (1995) *Journal of Biological Chemistry* **270**, 3662-3666

12. Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993) *Science* **260**, 88-91

13. Heller-Harrison, R.A., Morin, M., Guilherme, A., and Czech, M.P. (1996) *Journal of Biological Chemistry* **271**, 10200-10204

14. Karoor, V., Wang, L., Wang, H.Y., and Malbon, C.C. (1998) *Journal of Biological Chemistry* **273**, 33035-33041

15. Malbon, C.C. and Karoor, V. (1998) *Cellular Signalling* **10**, 523-527

16. Shih, M. and Malbon, C.C. (1998) *Cellular Signalling* **10**, 575-582

17. George, S.T., Berrios, M., Hadcock, J.R., Wang, H.Y., and Malbon, C.C. (1988) *Biochemical & Biophysical Research Communications* **150**, 665-672

18. Shih, M., Lin, F., Scott, J.D., Wang, H.Y., and Malbon, C.C. (1999) *Journal of Biological Chemistry* **274**, 1588-1595

19. Shih, M. and Malbon, C.C. (1994) *Proceedings of the National Academy of Sciences of the United States of America* **91**, 12193-12197

20. Shih, M. and Malbon, C.C. (1996) *Journal of Biological Chemistry* **271**, 21478-21483

21. Jho, E.H., Davis, R.J., and Malbon, C.C. (1997) *Journal of Biological Chemistry* **272**, 24468-24474

22. Gagnon, A.W., Kallal, L., and Benovic, J.L. (1998) *Journal of Biological Chemistry* **273**, 6976-6981

23. Karoor, V. and Malbon, C.C. (1996) *Journal of Biological Chemistry* **271**, 29347-29352

24. Karoor, V. and Malbon, C.C. (1998) *Advances in Pharmacology* **42**, 425-428
25. Bouvier, M., Chidiac, P., Hebert, T.E., Loisel, T.P., Moffett, S., and Mouillac, B. (1995) *Methods in Enzymology* 250, 300-314

26. Carpenter, C.L. and Cantley, L.C. (1996) *Current Opinion in Cell Biology* 8, 153-158

27. Galetic, I., Andjelkovic, M., Meier, R., Brodbeck, D., Park, J., and Hemmings, B.A. (1999) *Pharmacology & Therapeutics* 82, 409-425

28. Luttrell, L.M., Daaka, Y., and Lefkowitz, R.J. (1999) *Current Opinion in Cell Biology* 11, 177-183

29. Lazar, D.F., Wiese, R.J., Brady, M.J., Mastick, C.C., Waters, S.B., Yamauchi, K., Pessin, JE, Cuatrecasas, P., and Saltiel, A.R. (1995) *Journal of Biological Chemistry* 270, 20801-20807

30. Della, R.G., Mukhin, Y.V., Garnovskaya, M.N., Daaka, Y., Clark, G.J., Luttrell, LM, Lefkowitz, R.J., and Raymond, J.R. (1999) *Journal of Biological Chemistry* 274, 4749-4753

31. Della, R.G., van Biesen, T., Daaka, Y., Luttrell, D.K., Luttrell, L.M., and Lefkowitz, R.J. (1997) *Journal of Biological Chemistry* 272, 19125-19132
Table 1. Inhibition of PI3 kinase by LY294002 suppresses the sequestration of β2AR in response to insulin, but not in response to isoproterenol. A431 cells were treated in the absence (Control) and presence of either 10 µM isoproterenol or 100 nM insulin. The extent of β2AR sequestration was determined using the hydrophilic, cell-impermeable beta-adrenergic antagonist radioligand [³H]CGP-12177. The incubation with isoproterenol was for 30 min, whereas the incubation with insulin was measured at 5 min, as employed in the experiments shown in Figure 6. The isoproterenol-stimulated sequestration was blocked by addition of the beta-adrenergic antagonist propranolol (10 µM). Aliquots of cells were pretreated with the PI3K inhibitor LY294002 (20 µM) and then stimulated with either isoproterenol or insulin. The LY compound suppresses insulin-stimulated sequestration of the β2AR. The data are the mean values ± S.E.M. of three replicate experiments. Total binding was 29.4 ± 3.2 fmol/100,000 cells.

| Treatment | Sequestration of β2AR (%) |
|-----------|---------------------------|
| (30 min incubation) | |
| None (Control) | 0 |
| + Isoproterenol (10 µM) | 25.8 ± 1.8 |
| + Isoproterenol (10 µM) and propranolol (10 µM) | 0 |
| + Isoproterenol (10 µM) and LY294002 (20 µM) | 27.6 ± 2.4 |

| (5 min incubation) | |
| None (Control) | 0 |
| + Insulin (100 µM) | 19.5 ± 1.6 |
| + Insulin (100 nM) and LY294002 (20 µM) | 04.9 ± 0.7 |
Figure Legends

Figure 1. The activation of Erk1,2 in response to insulin is potentiated by β2AR. Chinese hamster ovary (CHO)-K cells and CHO clones stably transfected to express the β2AR (CHO-β2AR) were challenged with insulin (100 nM) for the time (min) indicated and the activation of Erk1,2 determined using antibodies specific for the dually phosphorylated, active forms of p44 and p42 Erk (phospho-Erk). The results of three independent assays in which the amount of the phospho-, activated forms of p44 and p42 Erk were quantified are summarized in the bar graphs.

Figure 2. The activation of Erk1,2 in response to insulin is increased with increasing expression of β2AR. CHO clones were stably transfected and selected to express different levels of β2AR, indicated as wild-type (WT, with very low expression of β2AR), low (βL), middle (βM), and high (βH) expressing clones. The activation of Erk1,2 was determined as indicated in legend to Figure 1 in clones incubated in the absence (-) or presence of either 100 nM insulin (+Insulin) or 50 ng/ml epidermal growth factor (+EGF) for 5 min. The results of three independent assays in which the amount of the phospho-, activated forms of p44 and p42 Erk were quantified are summarized in the bar graphs.

Figure 3. Activation of Erk1,2 in A431 cells: response to insulin and to isoproterenol. A431 cells were treated in the presence or absence of isoproterenol (10 µM) or insulin (100nM), or both for 5 min. Aliquots of untreated A431 cells as well as cells treated with either isoproterenol or with insulin were treated also with the beta-adrenergic antagonist propranolol (10 µM) to block beta-adrenergic stimulation by isoproterenol and any agonist-like activity derived from serum. To evaluate the activation of Erk1,2 in response to elevation of intracellular cyclic AMP, cells were treated with the diterpene forskolin (30 µM) for 5 min. The activation of
Erk1,2 determined using antibodies specific for the dually phosphorylated, active forms of p44 and p42 Erk (phospho-Erk). The results of three independent assays in which the amount of the phospho-, activated forms of p44 and p42 Erk were quantified are summarized in the bar graphs.

**Figure 4. Activation of Erk1,2 in A431 cells: dose-response to insulin and to isoproterenol, in combination.** A431 cells were treated in the presence or absence of isoproterenol (0, 1, or 10 µM) and insulin (0, 1, 10, or 100nM), or both for 5 min. The activation of Erk1,2 determined using antibodies specific for the dually phosphorylated, active forms of p44 and p42 Erk (phospho-Erk). The results of three independent assays in which the amount of the phospho-, activated forms of p44 and p42 Erk were quantified are summarized in the bar graphs.

**Figure 5. The activation of Erk1,2 in response to insulin is potentiated by β2AR and dependent upon the integrity of β2AR tyrosyl residue 350.** CHOK cells (CHOK) and clones expressing either the wild-type β2AR (Y350) or the tyrosine-to-phenylalanine (Y350F) mutant form of the β2AR were challenged with 100 nM insulin for 5 min and the amount of activated Erk1,2 determined. The results of three independent assays in which the amount of the phospho-, activated forms of p44 and p42 Erk were quantified are summarized in the bar graphs.

**Figure 6. The activation of Erk1,2 by insulin in epidermoid carcinoma A431 cells is blocked by expression of the C-terminal, cytoplasmic domain (BAC1) of the β2AR.** Panel A, A431 cells were stably transfected with an expression vector harboring the C-terminal, cytoplasmic domain (BAC1) of the hamster β2AR or the empty vector (-). Staining immunoblots of whole-cell extracts of A431 clones with antibodies against the C-terminal, cytoplasmic domain
of the β2AR reveals the expressed 11 kDa-Mr form of BAC1. Clones were challenged with 100 nM insulin for 5 min and the phosphotyrosyl-containing species identified in immunoblots of whole-cell extracts using an anti-phosphotyrosine antibody (Anti-pY). Note that the phosphotyrosine content of BAC1 is increased in A431 cells expressing BAC1 that have been challenged with insulin. Panel B, clones were challenged with 100 nM insulin for 5 min and the amount of the activated Erk1,2 was determined. Note that expression of BAC1 resulted in a block of the ability of insulin to activate Erk1,2 in these cells.

**Figure 7. Blockade of β2AR sequestration by the PI3K inhibitor LY294002.** A431 clones stably transfected to express GFP-tagged β2AR were subjected to confocal laser-scanning microscopy in the absence (a) or presence of 100 nM insulin (b,c). Note that the GFP-tagged β2AR is localized to the cell membrane in the absence of insulin. Insulin stimulates a sequestration of the GFP-tagged β2AR within 5 min. Treatment with the PI3K inhibitor LY294002 (20 µM) blocks the insulin-stimulated sequestration of the β2AR.

**Figure 8. The activation of Erk1,2 by insulin in epidermoid carcinoma A431 cells is blocked by inhibition of MEK with PD98059, but is not altered by the blockade of β2AR sequestration by the PI3K inhibitor LY294002.** Chinese hamster ovary (CHO)-K cells and CHO clones stably transfected to express the β2AR (CHO-β2AR) were challenged with insulin (100 nM) for 5 min and the activation of Erk1,2 determined using antibodies specific for the dually phosphorylated, active forms of p44 and p42 Erk (phospho-Erk). At 12 hr prior to the challenge with insulin, the clones were pretreated with or without one of three agents: 10 µM PD98059, 20 µM LY294002; or 50 nM PP2. The results shown in the bar graphs are representative of three independent assays.
Figure 9. The time-course and decay of activation of Erk1,2 by insulin in epidermoid carcinoma A431 cells is not altered by the blockade of β2AR sequestration by the PI3K inhibitor LY294002. Chinese hamster ovary (CHO)-K cells and CHO clones stably transfected to express the β2AR (CHO-β2AR) were challenged with insulin (100 nM) for 0, 3, 5, or 10 min and the activation of Erk1,2 determined using antibodies specific for the dually phosphorylated, active forms of p44 and p42 Erk (phospho-Erk). At 2 hr prior to the challenge with insulin, the clones were pretreated with or without 20 µM LY294002. The results shown in the bar graphs are representative of three independent assays.

Figure 10. Activation of Erk1,2 in A431 cells: analysis of insulin stimulation following a prior challenge with isoproterenol. A431 cells were treated challenged either with isoproterenol (0 or 10 µM) alone, insulin (0, 1, 10, or 100nM), or insulin (0, 1, 10, or 100nM) following a pretreatment with 10 µM isoproterenol. The activation of Erk1,2 determined using antibodies specific for the dually phosphorylated, active forms of p44 and p42 Erk (phospho-Erk). The results of three independent assays in which the amount of the phospho-, activated forms of p44 and p42 Erk were quantified are summarized in the bar graphs.

Figure 11. Activation of Erk1,2 in response to isoproterenol and to insulin. See text for details.
Fig. 1, Wang et al.

[Diagram showing phosphorylation of ERK in CHOK and CHO-β2AR cells with and without insulin treatment over time (0 to 5 minutes).]

- CHOK
- CHO-β2AR

Phospho-Erk
- p44
- p42

Phospho-p44Erk
Phospho-p42Erk

Density (arbitrary units)

Time (min)

+Insulin
Fig. 2 Wang et al.
Insulin
Isoproterenol
Propranolol
Forskolin

phosphoErk

phospho-p44Erk
phospho-p42Erk

Insulin
Isoproterenol
Propranolol
Forskolin
Ins(nM)  0  1  10  100  0  1  10  100  0  1  10  100
Iso(μM)  0  0  0  0  1  1  1  1  10  10  10  10

phospho-Erk:

**phospho-p44Erk**

**phospho-p42Erk**
Fig. 5 Wang et. al.

- -
CHOK Y350 Y350F

+ Insulin
CHOK Y350 Y350F

phosphoErk

p44
p42

phospho-p44Erk

phospho-p42Erk

Density (arbitrary units)

| Clones       | CHOK | Y350 | Y350F |
|--------------|------|------|-------|
| Control      |      |      |       |
| +Insulin, 5min |      |      |       |

Downloaded from http://www.jbc.org/ by guest on March 22, 2020
**Fig. 8 Wang et al.**

**Inhibitor**

-  PD  LY  PP2  -  PD  LY  PP2

**Insulin**

-  -  -  -  +  +  +  +

**phospho-Erk**

![phospho-Erk Image]

**phospho-p44Erk**

**phospho-p42Erk**

| Insulin | - | - | - | - | + | + | + | + |
|---------|---|---|---|---|---|---|---|---|
| Inhibitor | PD | LY | PP2 | PD | LY | PP2 | PD | LY | PP2 |

**Density (arbitrary unit)**

- 0  4  8  12  16  20

- 0  4  8  12  16  20
Fig. 9 Wang et al.

LY294002 (pre-incubate, 2hr)

Insulin (min)  
0  3  5  10  0  3  5  10

phospho-Erk

phospho-p44Erk

phospho-p42Erk

Density (arbitrary unit)

+Ins (min)  0  3  5  10  0  3  5  10

+L +LY294002
+ INS

IRTK

βAR

Grb2

+PI3K, Dyn

βAR-VA

Grb2

VA-βAR

Erk Activation

Tyr phosphorylation

LY-sensitivity

Ser, Thr phosphorylation

+ ISO

GRK

βAR

Arr

VA-βAR

Arr

Fig. 11 Wang et al.
Insulin activation of mitogen-activated protein kinases Erk1,2 is amplified via beta-adrenergic receptor expression and requires the integrity of the Tyr350 of the receptor

Hsien-yu Wang, Sergey Doronin and Craig C. Malbon

J. Biol. Chem. published online August 11, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004404200

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