Previous studies have shown that the endosomal apparatus plays an important role in insulin signaling. Inhibition of endosomal acidification leads to a decrease in insulin-insulin receptor kinase (IRK) dissociation and insulin degradation. Thus, vacuolar pH could function as a modulator of insulin signaling in endosomes. In the present study we show that in primary hepatocytes pre-treated with bafilomycin, there is an inhibition of vacuolar acidification. Incubation of these cells with insulin was followed by an augmentation of IRK activity but an inhibition of phosphorylidyinositol 3-kinase/Akt activity and a decrease in insulin-induced DNA and glycolgen synthesis. Bafilomycin treatment inhibited IRK recycling to the plasma membrane without affecting IRK internalization. Impaired IRK recycling correlated with a decrease in insulin signaling. We suggest that inhibiting vacuolar acidification sequesters activated IRKs in an intracellular compartment where signaling is inhibited. This implies that endosomal receptor trafficking plays a role in regulating signal transduction.

Insulin binding to its cell surface receptor (IRK) is followed by autophosphorylation, activation, and internalization of the IRK into endosomes (1). Although a proportion of IRK is targeted to lysosomes for degradation, most internalized IRK recycles to the plasma membrane (PM) (2, 3). Various studies have shown that signaling by growth factors and hormones, including insulin, occurs at both the cell surface and in endosomes (4–6). Indeed the exclusive activation of endosomal IRK (5) and epidermal growth factor receptor (7) has been shown to yield substrate tyrosine phosphorylation and signal transduction. It follows that the extent and duration of cell signaling is subject to modulation by endosomal processes (3, 8). It has been demonstrated that the endosomal IRK is dephosphorylated by an associated phosphotyrosine phosphatase (3, 9). Furthermore, a reduced intraendosomal pH (pH ≤ 6) promotes dissociation of internalized insulin-IRK complexes and degradation of insulin (10, 11) by an acidic endosomal insulinase recently identified as cathepsin D (12). The acidic pH of endosomes also effects a conformation-dependent inactivation of the IRK (13). Thus, the acidic pH of the endocytic compartment and the IRK-associated phosphotyrosine phosphatase(s) therein play fundamental roles in attenuating insulin signaling.

Weak bases, proton ionophores, and inhibitors of vacuolar ATPases have been used to study the role of acidification in physiological processes (14, 15). In liver, chloroquine (a weak base) accumulates in hepatic endosomes leading to the inhibition of both the dissociation of insulin-IRK complexes and insulin degradation (16–18). This suggests that inhibitors of vacuolar acidification would potentiate insulin signaling from the endosomal compartment, and there are studies consistent with this view. Thus, in rats, chloroquine treatment augmented and prolonged IRK activity in hepatic endosomes (19). Further, the administration of chloroquine to patients with type 2 diabetes mellitus decreased fasting plasma glucose levels, improved glucose tolerance, and increased plasma insulin levels (20–22). However, it is not clear whether the effects of chloroquine in these patients are associated exclusively with improved insulin sensitivity as opposed to an increase in plasma insulin levels. In contrast, a previous study in adipocytes showed that chloroquine inhibited insulin-induced GLUT4 translocation to the PM (23). In view of the importance of this issue to an understanding of insulin signaling and potentially insulin resistance and in view of the above-noted discrepancies, we examined the role of vacuolar acidification on insulin signaling in primary rat hepatocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purine insulin (24.5 units/mg) was a gift from Lilly Research Laboratories (Indianapolis, IN). Collagenase was purchased from Worthington Biochemical Corp. (Freehold, NJ). Cell culture medium and antibiotics were obtained from Invitrogen, and Vitrogen-100 was obtained from Collagen Corp. (Toronto, Canada). [3H]Methylthymidine (20 Ci/mmol) was obtained from ICN Biomedicals, Inc., Canada Ltd. (Mississauga, Canada). ATP was purchased from Roche Applied Science. [U-14C]Glucose (300 mCi/mmol) and [γ-32P]ATP (3000 Ci/ mmol) were purchased from PerkinElmer Life Sciences. The antibodies against Akt and phospho-Akt (Ser473) were purchased from New England Biolabs, Inc. (Mississauga, Canada). An antibody against tyrosine-phosphorylated proteins (PY99) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phos-IRS1, anti-IRS2, and anti-phospho-GSK-3 (Ser21) antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). An antibody raised against a peptide corresponding to residues 942–969 of the IRK β-subunit (a960) was used for Western blotting and prepared as previously described (25). For immunoprecipitation of IRK, an antibody directed against the α-subunit was obtained from the serum of a patient with acanthosis nigricans (1). Protein A-Sepharose was obtained from Amersham Biosciences. Immobilon-P transfer membranes were obtained from Millipore Corp. Canada Ltd. (Mississauga, Canada). Bafilomycin A1, Chol, Chol, poly(GluTyr), and most other chemicals were purchased from Sigma.

**Cell Culture**—Primary hepatocytes isolated from 120–140-g male Sprague-Dawley rats (Charles River Laboratories, Inc., St. Constant, Canada) in situ liver perfusion with collagenase were plated on a collagen matrix (Vitrogen-100). Subconfluent cultures were prepared by
seeding 1 × 10^6 cells, onto 9.6-cm² six-well plates (Corning, Costar, Cambridge, MA) or 5 × 10^6 cells, onto 78-cm² culture dishes (Starstedt Canada, St. Laurent, Canada). The cells were bathed for 24 h in seeding medium (Dulbecco's modified Eagle's medium/Ham's F-12 containing 10% fetal bovine serum, 10 mM HEPES, 20 mM NaHCO₃, 500 IU/ml penicillin, and 500 µg/ml streptomycin) and then for 4 h in serum-free medium that differed from the seeding medium in that it lacked fetal bovine serum and contained 1.25 µg/ml fungizone, 0.4 mM ornithine, 2.25 µg/ml L-lactic acid, 2.5 × 10⁻⁸ M selenium, and 1 × 10⁻⁸ M ethanolamine. Serum-free medium was renewed before the addition of [³H]thymidine, insulin, and bafilomycin.

Glycogen Synthesis—Glycogen synthesis was determined by incorporation of [U-¹⁴C]glucose into glycogen as previously described (26). Briefly, the hepatocytes (1×10⁶ cells) were serum-deprived for 4 h and incubated for 2 h in serum-free medium containing 15 mM [U-¹⁴C]glucose in the presence or absence of bafilomycin A1 (100 nM) and insulin (100 nM). Incubations were stopped by three rapid washes with ice-cold PBS, and the cells were solubilized in 1 ml of 0.1 M NaOH. The samples were then boiled in the presence of 2 mg of carrier glycogen and precipitated overnight in 70% ethanol at -20 C. After centrifugation, the precipitated glycogen was resuspended in 500 µl of water and incubated for 5 min at 70 °C. Incorporated radioactivity was determined by scintillation counting as previously described (27).

Thymidine Incorporation Assay—Subconfluent hepatocytes were plated on 9.6-cm² six-well plates in serum-containing medium for 24 h and then in serum- and growth factor-free medium for an additional 48 h. Thymidine (5 µCi/ml), insulin, and bafilomycin A1 were added to cells as described in the figure legends. After an 18-h incubation, the cells were rinsed twice with 3 ml of cold PBS, incubated for 15 min at 4 °C in 10% trichloroacetic acid, solubilized at room temperature in 1 ml of 1 N NaOH, and then transferred to scintillation vials for counting of [³H] in an LKB liquid scintillation counter as described before (24).

Preparation of Cell Lysates—Primary rat hepatocytes were rinsed twice with ice-cold phosphate-buffered saline, pH 7.4, and solubilized with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 100 µM sodium fluoride, 1.5 mM MgCl₂, 1 mM EGTA, 200 µM sodium orthovanadate, 1 mM phenethylisothiourea, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10% glyceral, and 1% Triton X-100). The cell lysates were clarified by centrifugation at 10,000 × g for 20 min at 4 °C, and protein concentration was determined in the resulting supernatant.

PI 3-Kinase Activity—Phosphotyrosine proteins were immunoprecipitated from cell lysates with an aPY antibody. The immunoprecipitates were extensively washed, and the protein A-Sepharose pellet was resuspended in 50 µl of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) containing 0.5 mg/ml phosphatidylinositol and assayed for PI 3-kinase activity as previously described (28).

Measurement of Exogenous IRK Activity—IRK from cell lysates were partially purified on wheat germ agglutinin columns as previously described (5). Exogenous tyrosine kinase activity was determined using poly(Glu:Gly:5:1) as substrate (27).

Determination of the Level of IRK Associated with the PM—Detection of PM-IRK was performed as described previously (29). Briefly, hepatocytes were incubated in the presence or absence of bafilomycin and insulin as noted in figure legends. Thereafter, hepatocytes were washed three times with ice-cold PBS-Ca²⁺-Mg²⁺ (PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂, pH 7.4), and cell surface proteins were biotinylated by incubation with 0.5 mg/ml of Sulfo-NHS-LC-Biotin (Pierce) in PBS-Ca²⁺-Mg²⁺ containing 15 mM of glycine. After biotinylation, the cell lysates were prepared as described above, and IRK was immunoprecipitated with an antibody directed against the α-subunit of the IRK. The immunoprecipitates were boiled in the presence of Laemmli buffer and subjected to SDS-PAGE. The proteins were

![Image](image-url)
transferred to Immobilon-P membranes and immunoblotted with α960 or streptavidin-horseradish peroxidase (Amersham Biosciences). Streptavidin binds to biotinylated proteins, allowing only the detection of IRK associated with the PM.

**Immunofluorescence**—Acidic compartments in primary hepatocytes were visualized by immunofluorescence with the DAMP method as indicated by the manufacturer (Oxford Biomedical Research, Oxford, MI). Briefly, the primary hepatocytes were incubated with DAMP (30 µM) for 30 min, fixed in 4% paraformaldehyde, washed, and permeabilized by incubation with 0.5% Triton X-100. The fixed hepatocytes were incubated with a monoclonal anti-DNP antibody for 60 min and subsequently washed to remove unbound IgG. The cells were then incubated for 60 min with anti-mouse IgG conjugated to rhodamine and viewed under a Zeiss Axiovert 135 confocal microscope. Photographs were taken using a Zeiss LSM microsystem.

**Preparation of Endosomes**—Primary hepatocytes from seven culture dishes (78 cm²) were homogenized in 12 ml of ice-cold sucrose buffer (5 mM Tris-HCl buffer, pH 7.4, 0.25 M sucrose, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl₂, 2 mM NaF, and 2 mM orthovanadate). The endosomes were prepared as previously described (1).

**RESULTS**

Bafilomycin is a macrolide antibiotic that, at concentrations up to 1 μM, specifically inhibits vacuolar ATPases without the side effects induced by proton ionophores and weak bases (15, 30). In this study we use bafilomycin to evaluate the effect of inhibiting vacuolar acidification on insulin signaling.

**Effect of Bafilomycin on Vacular Acidification and Trafficking of IRK**—First, we confirmed that bafilomycin inhibits vacuolar acidification by incubating primary hepatocytes with DAMP, a compound that accumulates in acidic vesicles. On performing this incubation, DAMP, detected by immunofluorescence (see "Experimental Procedures"), was found in granular structures in the presence or absence of insulin (Fig. 1, top panels). Preincubation with bafilomycin (100 nM for 30 min) abolished the granular fluorescence almost completely (Fig. 1, top panels), indicating that bafilomycin inhibited vacuolar acidification in primary hepatocytes.

Previous work has shown that inhibition of vacuolar acidification...
acation interferes with receptor recycling (31, 32). We used cell surface biotinylation to assess the effect of bafilomycin on insulin-induced IRK internalization and recycling to the cell surface. Hepatocytes were preincubated with insulin (100 nM) in the presence or absence of bafilomycin, and cell surface proteins were subsequently labeled with Sulfo-Biotin as described under “Experimental Procedures.” The cells were lysed, the IRKs were immunoprecipitated from cell lysates, and both total and PM-associated IRKs were detected by immunoblotting with 9251 and streptavidin, respectively. It can be seen that bafilomycin did not inhibit insulin-induced IRK internalization (Fig. 2a). Consistent with this was our observation that, following 15 min of incubation with insulin, the increase in the level of IRK in a crude endosomal preparation from hepatocytes was not significantly affected by bafilomycin (analysis by Student’s t test, p < 0.05; data not shown).

To investigate the effect of bafilomycin on IRK recycling, internalization of IRK was promoted by incubating hepatocytes with insulin (100 nM) for 10 min followed by washing to remove ambient insulin and further incubation for 10 min to allow recycling of internalized IRK. In control cells most internalized IRK recycled to the PM in the absence of ambient insulin, whereas in cells incubated with bafilomycin recycling of IRK to the PM was strikingly inhibited (Fig. 2b).

Effect of Bafilomycin on Insulin Action in Primary Hepatocytes—To determine the effect of bafilomycin on insulin action, we measured the effect of insulin on thymidine incorporation and glycogen synthesis in hepatocytes incubated with or without bafilomycin. As is evident in Fig. 3, bafilomycin significantly inhibited the effect of insulin on both thymidine incorporation and glycogen synthesis by 40 and 60%, respectively.

Fig. 5. Bafilomycin inhibits insulin-induced tyrosine phosphorylation of IRS2 but not its association with p85. Primary hepatocytes were treated with bafilomycin (100 nM) for 30 min at 37°C followed by insulin (100 nM) or buffer for 2 or 15 min. IRS1 and IRS2 were immunoprecipitated (IP) from lysates (1 mg of protein) with specific antibodies bound to protein A. The immunoprecipitates were washed, boiled in Laemmli buffer, and subjected to SDS-PAGE, and the proteins were transferred to Immobilon-P membranes as described under “Experimental Procedures.” The level of phosphotyrosine (a) and p85 (b) associated with IRS1 and IRS2 were determined by immunoblotting with 9251 and p85, respectively. A representative immunoblot at the top of each bar graph is shown. The results are the means ± S.E. of three to five separate experiments. *, p < 0.005 (versus 15 min of insulin). PY, phosphotyrosine; WB, Western blot.
FIG. 6. Bafilomycin inhibits insulin-induced PI 3-kinase activity. a, hepatocytes were treated with or without bafilomycin (100 nM) for 30 min at 37 °C, after which insulin (100 nM) was added for 2 or 15 min. PI 3-kinase activity was measured in αIRS1 and αIRS2 immunoprecipitates (IP) with [γ-32P]ATP as described under “Experimental Procedures.” An autoradiograph depicting the phosphorylation of phosphatidylinositol in position 3 from an experiment performed in duplicate is shown. b, hepatocytes were treated as indicated above, and PI 3-kinase activity was measured in αPY immunoprecipitates. A representative autoradiograph is shown. Autoradiographs were quantified by scanning densitometry, and the results are expressed as the means ± S.E. of three separate experiments, each performed in triplicate. *, p < 0.01 (versus 15 min of insulin). c, the p85 subunit of PI 3-kinase was immunoprecipitated from hepatocyte lysates (1 mg of protein) with an αp85 antibody. The immunoprecipitates were washed, boiled in Laemmli buffer, and subjected to SDS-PAGE, and the proteins were transferred to Immobilon-P membranes as described under “Experimental Procedures.” Immunoblotting was performed with αPY. A representative immunoblot (one of four separate experiments) is shown. To assess the specificity of Immunoprecipitation, control samples were immunoprecipitated with normal IgG. WB, Western blot.
IRS2 phosphorylation by 35% ($p < 0.005$ versus insulin alone) in hepatocytes stimulated with insulin for 15 min (Fig. 5a, right panel).

In response to insulin, tyrosine phosphorylated IRS1 and IRS2 bind p85 (a regulatory subunit of PI 3-kinase), leading to the activation of PI 3-kinase. We thus assessed the effect of bafilomycin pretreatment on the extent of association of p85 with IRS1 and IRS2 following insulin treatment. As seen in Fig. 5b, incubation of hepatocytes with insulin stimulated the association of p85 with both IRS1 and IRS2 immunoprecipitates. Pretreatment with bafilomycin had no effect on the extent of the insulin-induced association (Fig. 5b). We then determined whether insulin-stimulated activation of PI 3-kinase associated with IRS1 or IRS2 is affected by bafilomycin. As seen in Fig. 6a, bafilomycin had no effect on PI 3-kinase activation assessed in anti-IRS1 and -IRS2 immunoprecipitates at 2 min but reduced this activity by 80% in anti-IRS2 immunoprecipitates at 15 min following incubation with insulin. Next we investigated the impact of reduced IRS2 associated PI 3-kinase activity on the total activity. Bafilomycin had no effect on PI 3-kinase activation assessed in anti-phosphotyrosine immunoprecipitates at 2 min but reduced this activity significantly by 60% at 15 min ($p < 0.01$ versus insulin alone) following incubation with insulin. As seen in Fig. 6a, a broad band at 160–180 kDa represents the main phosphotyrosine protein co-immunoprecipitated with p85 in response to insulin. Previous studies in hepatocytes have shown that this band corresponds almost exclusively to IRS1 and IRS2 (33). Thus, in hepatocytes pretreated with bafilomycin, IRS2 is probably the main tyrosine-phosphorylated protein involved in the decrease of PI 3-kinase activity. To a minor extent, insulin increased the association of a 60-kDa phosphotyrosine protein with p85 (Fig. 6c), but bafilomycin did not cause a significant change in this association (four independent experiments; analysis by Student’s t test, $p > 0.05$ versus insulin alone). Also, 110–115- and 200-kDa phosphotyrosine proteins associated with p85 (Fig. 6c), but such an association was not increased in response to insulin. In conclusion, bafilomycin increased IRK tyrosine kinase activity but paradoxically decreased PI 3-kinase activity associated with phosphotyrosine proteins in hepatocytes 15 min after insulin. Such a decrease correlated with diminished tyrosine phosphorylation of IRS2 and decreased PI 3-kinase activity associated with IRS2.

Insulin treatment results in a PI 3-kinase-dependent activation of Akt by phosphorylation at specific Ser residues, in particular Ser$^{473}$ (34). We therefore determined the effect of bafilomycin on Akt activation by immunoblotting with antibody specific for Akt phosphorylated at Ser$^{473}$. Pretreatment with bafilomycin, in parallel with its effect upon PI 3-kinase, did not affect Akt-Ser$^{473}$ phosphorylation at 2 min but inhibited
it by 40% \((p < 0.0001 \text{ versus insulin alone})\) at 15 min after insulin (Fig. 7a). Furthermore, bafilomycin inhibited Akt-Ser\(^{473}\) phosphorylation by 50–60\% with respect to corresponding controls at 0.5, 2, and 4 h after insulin (Fig. 7b). These latter results correlate with the inhibitory effect of bafilomycin on insulin-stimulated glycogen synthesis and thymidine incorporation, both of which determinations entailed longer incubations with insulin (see “Experimental Procedures”). There was no effect of bafilomycin on the level of hepatocyte Akt as determined by immunoblotting (data not shown).

In response to insulin, Akt activation results in augmented phosphorylation of its substrate, GSK-3, with accompanying inactivation of this enzyme, which contributes to insulin-induced activation of glycogen synthase (35). We employed immunoblotting to assess the extent of phosphorylation at Ser\(^{21}\) and Ser\(^{9}\) of GSK-3\(\alpha\) and GSK-3\(\beta\), respectively, as a measure of insulin-induced GSK-3 inactivation. In parallel with our observations on PI 3-kinase and Akt, bafilomycin did not affect GSK-3 serine phosphorylation at 2 min after insulin but inhibited it by 20\% \((p < 0.05 \text{ versus insulin alone})\) at 15 min after insulin (Fig. 7c).

Bafilomycin neutralizes endosomal acidification by inhibiting vacuolar ATPases responsible for maintaining a proton gradient (15). It was therefore of interest to determine whether chloroquine, an acidophilic compound shown to neutralize vacuolar pH by virtue of being a weak base (36), would similarly affect insulin signaling. The preincubation of hepatocytes with 0.1 mM chloroquine increased IRK activity and decreased Akt-Ser\(^{473}\) phosphorylation at 15 but not at 2 min after insulin (Fig. 8). Chloroquine at 1.0 mM promoted an even greater increase of IRK activity at 15 min after insulin but also increased IRK tyrosine phosphorylation at this time (Fig. 8, a, lower panel, and b). At this higher dose of chloroquine we observed an inhibition of Akt-Ser\(^{473}\) phosphorylation at both 2 and 15 min after insulin (Fig. 8c). These results raise the possibility that high concentrations of chloroquine may affect signaling by a mechanism additional to that of vesicular acidification as previously suggested (23, 37). In summary, the similarity between the effects of bafilomycin and chloroquine (0.1 mM) on IRK and Akt activation suggests that bafilomycin affects insulin signaling by disrupting vesicular acidification.

**Relation of Akt Activation to IRK Recycling**—The mechanism by which bafilomycin inhibits insulin signaling at 15 min, but not at earlier times of exposure, could involve an increase in the kinetics of PI 3-kinase/Akt inactivation after maximal activation or a reduction in surface IRK because of the inhibition of
IRK recycling. To differentiate between these two possible mechanisms, we studied in more detail the time course of insulin-stimulated Akt-Ser<sup>473</sup> phosphorylation. As seen in Fig. 9a, incubation with insulin resulted in maximal phosphorylation of Akt between 2 and 5 min, a rapid decrease at 8 min, and a subsequent increase at 15 min. Pretreatment with bafilomycin resulted in an insulin-stimulated time course of Akt-Ser<sup>473</sup> phosphorylation, which was greater than control between 2 and 8 min (difference at 8 min, \( p < 0.05 \)) but significantly inhibited thereafter (Fig. 9a). These results suggest that, in the presence of ambient insulin, the recycling of IRK is accompanied by a second wave of PI 3-kinase/Akt activation (24, 38).

Paradoxically, we found that the bafilomycin-induced decrease in PI 3-kinase/Akt activity was accompanied by a corresponding augmentation of IRK activity, and both of these effects of bafilomycin were observed only at later times (15 min) after insulin stimulation. They propose that the different effects of bafilomycin on insulin signaling at early and late times reflect differences in the cellular location of IRK at 2 and 15 min after insulin stimulation. The lack of an inhibitory effect of bafilomycin on insulin signaling at 2 min reflects the high proportion of IRK at the PM (Fig. 2a) and in early endosomal elements (25) at this time. In contrast, the effects of bafilomycin on IRK activity and signaling at 15 min after insulin coincide with the intracellular accumulation of receptor (Fig. 2a). Bafilomycin did not interfere with the insulin-stimulated internalization of IRK but inhibited the recycling of IRK to the PM (Fig. 2). This is consistent with the view that IRK is being sequestered into an intracellular subcompartment where signaling is blunted. This study also shows that IRK recycling is critical for PI 3-kinase/Akt activation (Fig. 9b), leading us to conclude that continuous recycling of IRK between internal compartments and PM is necessary for sustained insulin signaling.

The comparable effect of chloroquine to augment insulin-dependent IRK activation while inhibiting Akt activation at later times after insulin stimulation (Fig. 8) argues that these responses are consequences of the inhibition of vacuolar acidification. Interestingly at higher doses other effects of chloroquine were observed including the inhibition of Akt activity at 2 min after insulin. We suggest that this could result from nonacidotropic effects of chloroquine such as its capacity, at higher concentrations, to effect vacuolar swelling (15).

Previous work has shown that bafilomycin inhibited the trafficking from late endosomes to lysosomes of the fluid phase marker, horseradish peroxidase (39). Also, we have previously shown that chloroquine promotes the accumulation of insulin in a late endosomal fraction derived from liver (16). In hepatocytes, late endosomes are filled with tracers between 10 and 15 min (40). Thus, in the presence of bafilomycin, activated IRK could be sorted into the luminal vesicles of late endosomes and thereby segregated away from the cytoplasm, preventing interactions with downstream signaling proteins (41).
of association of p85 with IRS2. A possible explanation for this observation could be that the diminished phosphorylation of IRS2 did not affect the recruitment of p85 but did affect the association of the SH2 domains of p85 with IRS2 leading to decreased PI 3-kinase activity. In agreement with this, it has been shown that full activation of PI 3-kinase by tyrosine-phosphorylated proteins required occupancy of both viral SH2 domains in p85 (42).

In previous work we showed that inhibition of endosomal acidification with chloroquine inhibited both insulin degradation and dissociation of insulin-IRK complexes within endosomes (18), with the consequent augmentation of IRK activity in this compartment (19). Because a critical role for endosomal IRK in insulin signaling has been clearly demonstrated (5), it was postulated that the inhibition of vacuolar acidification might potentiate insulin signaling from endosomes (19). Our results support this view at early times following insulin administration. Thus, in the present study, bafilomycin significantly increased Akt activity when hepatocytes were incubated with insulin for 8 min (Fig. 8a). This suggests that bafilomycin augmented insulin signaling from early endosomes and that the subsequent accumulation of IRK into a nonfunctional "compartment" resulted in the attenuation of insulin signaling at 15 min. Our finding is in agreement with previous studies where it has been shown that bafilomycin abridged proliferation of various cell lines (43) as well as mitogen-induced DNA synthesis in 3T3 fibroblasts (44). Of great interest is the study showing inhibition in endosomes of hormone/growth factor dissociation sequences for the pathogenesis of disease.

In summary, we have found that the inhibition of endosomal acidification and chloride influx blocked insulin receptor recycling via the recycling of IGF-1 receptors in response to IGF-1 but inhibited both the recycling of IGF-1 receptors to PM and IGF-1-induced DNA synthesis (45). These observations together with our findings suggest that the inhibition in endosomes of hormone/growth factor dissociation from its receptor results in the prevention of receptor recycling, sequestration of the complex, and inhibition of signaling.

Several observations from a clinical setting may be relevant to this idea of inactivation of receptor function through sequestration of receptors. Thus, transformed lymphoblasts from subjects with type 2 diabetes mellitus showed impaired intracellular dissociation of insulin-IRK complexes, insulin degradation, and recycling of the IRK when compared with cells from normal subjects, raising the possibility that these defects might underly the insulin resistance seen in these patients (46). Also it has recently been observed that the sulfonflies glimepiride improved insulin action in hepatoma cells and correspondingly increased the intracellular dissociation of insulin-IRK complexes, the degradation of insulin, and the recycling of internalized IRK in these cells (47). It is tempting to consider that a defect in the vascular system responsible for mediating insulin action (endosomes) and insulin processing and secretion (trans Golgi vesicles) may explain both the insulin resistance and impaired β cell function of type 2 diabetes mellitus.

In summary, we have found that the inhibition of endosomal acidification blocks IRK recycling and reduces insulin signaling at later times. We propose that this arises as a result of the sequestration of activated receptors such that they no longer can influence downstream signaling elements. Thus, a continuous trafficking of IRK between endosomes and PM is required to sustain insulin signaling. These observations suggest that disturbances in the trafficking of IRK and possibly other receptors within the endosomal system may have important consequences for the pathogenesis of disease.